Actinobacteria and *Myxobacteria* isolated from freshwater snails and other uncommon Iranian habitats, their taxonomy and secondary metabolism

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Tagungsbeiträge

Actinobacteria and myxobacteria isolated from freshwater snails

(Talk in 11th Annual Retreat, HZI, 2020)

Posterbeiträge

- Myxobacteria and Actinomycetes isolated from freshwater snails and other uncommon Iranian habitats, their taxonomy and secondary metabolites

(11th and 12th International PhD Symposium, HZI Braunschweig, 2018 and 2019)

- Taxonomy of a novel Actinobacteria isolated from Iranian soil sample

(8th and 9th International PhD Symposium, HIPS Saarbrücken, 2018 and 2019)

- Poly-phasic taxonomy of a novel Acinobacteria isolated from soil sample

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“May the gratitude in my heart, kiss all the universe.”

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List of Abbreviations

MDR	Multidrug resistant
VDR	Vancomycin-resistant enterococci
MRSA	Methicillin-resistant *Staphylococcus aureus*
ESBL	Extended-spectrum β-lactamases
HTS	High-throughput screening
GSK	GlaxoSmithKline
DNP	Dictionary of Natural Products
Mbp	Millions of base pairs
ISP	International *Streptomyces* project
SEM	Scanning electron microscopy
DAP	Diaminopimelic acid
DDH	DNA-DNA hybridization
G+C	Guanine and cytosine
GGDC	Genome to Genome Distance Calculator
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
ANI	Average Nucleotide Identity
TYGS	Type (Strain) Genome Server
HV	Humic Acid-Vitamin
ABL	Advanced Biological Laboratories
MIC	Minimal inhibition concentration
RPM	Revolutions per minute
°C	Degree Celsius
GYM	Glucose–Yeast–Malt extract medium
MP	Maximum-Parsimony
ML	Maximum-Likelihood
JSRM	Jump start ready mix
PCR	Polymerase chain reaction
HPLC	High performance liquid chromatography
HR-ESI-MS	High resolution electron spray ionization mass spectrometry
DAD	Diode-Array Detection
MHB	Mueller Hinton Broth
UPLC	UltraPerformance Liquid Chromatography
SSM+T	Synthetically Suter medium with tyrosine
SSM-T	Synthetically Suter medium without tyrosine
MLSA	Multilocus sequence analysis
LC-MS	Liquid chromatography – mass spectrometry
NMR	Nuclear magnetic resonance
PKS	Polyketide synthases
NRPS	Nonribosomal peptide synthetases
TIC	Total ion current

Abstract

A dramatic reduction in the discovery of new antimicrobial compounds has led researchers to search for rare antibiotic-producing species in unexplored habitats. Two group of Actinobacteria and Myxobacteria are known as rich sources for bioactive secondary metabolites. In order to find rare and novel species of these two groups, some under-investigated habitats like the deserts, mangrove forests, beach sands, the soil of medicinal plants and freshwater snails were chosen as sources for isolation. Totally, 108 actinobacterial strains and 36 myxobacterial strains were isolated from soil samples (80 isolates of Actinobacteria, 32 isolates of Myxobacteria) and from different genera of freshwater snails (28 isolates of Actinobacteria, 4 isolates of Myxobacteria). Whole-genome sequencing identified six novel species of Actinobacteria belonging to the genera *Kibdelosporangium* (4NS15), *Amycolatopsis* (Q1), *Pseudonocardia* (Q2), *Sanguibacter* (NS44ZA), *Agromyces* (BA) and *Streptomyces* (7NS3). Analyses of the extracts from two isolates 4NS15 and 7NS3 showed good antibacterial activity. Strain 4NS15, isolated from desert soil could produce a new family of compounds belonging to the class of terpenoids. The name *Kibdelosporangium persicum* sp. nov has been proposed for this new representative of the genus *Kibdelosporangium.* A metagenomic approach using cultivation-independent high-throughput sequencing revealed that the phylum Actinobacteria in the freshwater snail *Physa acuta* represented only 2% of the bacterial community. However, an isolated strain 7NS3 from this freshwater snail was detected as a novel *Streptomyces* species. Bioassay-guided high performance liquid chromatography (HPLC) and high-resolution electrospray ionization-mass spectrometry (HR-ESI-MS) analyses of its crude extract fractions resulted in the detection of a compound, which showed match characteristics with the already known compound emycin A, an angucycline aromatic polyketide. Our study demonstrates that with targeting unexplored habitats, we can still isolate novel species that potentially produce novel secondary metabolites.

Keywords: Rare Actinobacteria, Iran, unexplored habitats, freshwater snails, *Physa acuta*

Abstrakt

Ein drastischer Rückgang bei der Entdeckung neuer antimikrobieller Substanzen veranlasste Forscher dazu, nach seltenen Antibiotika-produzierenden Arten in noch unerschlossenen Habitaten zu suchen. Zwei Gruppen von Actinobakterien und Myxobakterien sind als ergiebige Quellen für bioaktive Sekundärmetabolite bekannt. Um neue und seltene Spezies dieser zwei Gruppen zu finden, wurden einige bislang wenig untersuchte Lebensräume wie die Wüste, Mangrovenwälder, Strandsand, Heilpflanzen und Süßwasserschnecken als Quellen für die Identifizierung ausgewählt. Insgesamt wurden 108 Actinobakterienstämme und 36 Myxobakterienstämme aus Bodenproben (80 Isolate von Actinobakterien, 32 Isolate von Myxobakterien) und aus verschiedenen Gattungen von Süßwasserschnecken (28 Isolate von Actinobakterien, vier Isolate von Myxobakterien) isoliert. Durch vollständige Genomsequenzierung konnten sechs neue Spezies der Actinobakterien, welche zu den Gattungen *Kibdelosporangium* (4NS15), *Amycolatopsis* (Q1), *Pseudonocardia* (Q2), *Sanguibacter* (NS44ZA), *Agromyces* (BA) and *Streptomyces* (7NS3) gehören, identifiziert werden. Die Analyse von Extrakten zweier dieser Isolate zeigte gute antibakterielle Aktivität. Stamm 4NS15, isoliert aus Wüstenboden, konnte eine neue Gruppe von Verbindungen produzieren, die zur Familie der Terpenoids gehören. Als Name für diesen neuen Vertreter der Gattung *Kibdelosporangium* wurde *Kibdelosporangium persicum* sp. nov vorgeschlagen. Ein metagenomischer Ansatz mit kultivierungsunabhängiger Hochdurchsatzsequenzierung ergab, dass das Phylum Actinobacteria in der Süßwasserschnecke *Physa acuta* nur zwei Prozent der bakteriellen Population ausmacht. Jedoch wurde der aus dieser Süßwasserschnecke isolierte Stamm 7NS3 als neue *Streptomyces*-Spezies identifiziert. Wirkorientierte Hochleistungsflüssigkeitschromatographie (bioassay-guided high performance liquid chromatography, HPLC) und hochauflösende Elektrospray-Ionisations-Massenspektrometrie (HR-ESI-MS)-Analysen seiner Rohextraktfraktionen führten zum Nachweis einer Verbindung, die Übereinstimmungen mit der bereits bekannten Verbindung Emycin A, einem angucyclin-aromatischen Polyketid, aufwies. Unsere Studie zeigt, dass wir mit der gezielten Suche in unerforschten Habitaten immer noch neue Arten isolieren können, die möglicherweise neue Sekundärmetaboliten produzieren.

Stichworte: Seltene Actinobakterien, Iran, unerforschte Habitate, Süßwasserschnecken, *Physa acuta*

1 Introduction

1.1 Antibiotics and natural products

The invention of microscope by van Leeuwenhoek, yielded in identification of bacteria in the 1670s but the connection between bacteria and diseases found in nineteenth century by Robert Koch (1). As a result, one of the fundamental accomplishments of human kind in the history of medicine was the discovery of antibacterial substances (2). The term "Antibiotics" was first used by Selman Waksman in 1941 and described a small molecule produced by microorganisms with antagonistic characteristic on the other microbe growth (3). This definition of antibiotics was brought in 13 years after the first observations of penicillin (3). The accidental observation of Alexander Fleming on the growth inhibition of Staphylococci by mold colonies in 1928, had a great impact on the future of antimicrobial discovery (4). After eleven years from Fleming's discovery, three scientists named Howard Florey, Norman Heatly and Ernst Chain could purify and scale up penicillin for clinical usage in 1939 (4). The revolution of human medicine and the path towards golden era, started by Waksman research on soil (5). Waksman and his team discovered the significance impact of soil bacteria (actinomycetes) on inhibiting the growth of other bacteria in a competitive atmosphere that leads to introducing the concept of 'Waksman platform' in 1938 and the definition of antibiotics in 1941 (4). Waksmans team screened soil-derived streptomycetes for antimicrobial activity by detecting inhibition of growth zones on plates and this work was adopted by the pharmaceutical industry and the results initiated 'the antibiotic golden era' (6).

Over the next 20 years (1940s until 1960s), the major classes of antibiotics were discovered and one-half of them are still in clinical use (Figure 1-1). Although many of the discovered antibiotics saved the patient's lives (especially the role of penicillin on treatment of the infections from second world war soldiers), shortly thereafter antibiotic resistance crisis happened (7). Unrestricted use of antibiotics and the reduction in antibiotic discovery from the 1970s brought the need of finding new antibiotics. Two main reasons eliminated the golden age of antibiotic discovery: First, easy-to-find antibiotics had been discovered and for finding new classes of antibiotics, scientists required more effort and clever thinking. Second, from commercial point of view, the

benefit of selling antibiotics for pharmaceutical companies was low and they lost their interest in new investments, because new antibiotics have been used just in serious infections (2).

Entering next era known as antibiotic medicinal chemistry era from mid 1960s to 2000s started with a gap in finding new antibiotics by Waksman platform and caused designing semi-synthetic and entirely synthetic antibiotics (4,8). Although finding the new semi-synthetic derivatives was an outstanding improvement but it brought next era, which lasted until now and we have known it as era of antibiotic resistance (8). Specialists are worried that we return to the pre-antibiotic era due to spread of multidrug resistant (MDR) strains (9). Multidrug-resistant (MDR) pathogens, like carbapenem-resistant *Pseudomonas aeruginosa*, vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), and extended-spectrum β-lactamases (ESBL) are of the most problematic for healthcare (9).

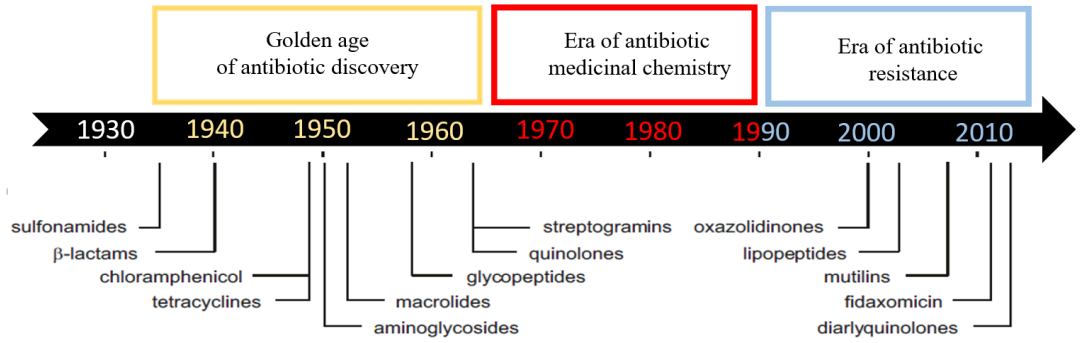


Figure 1-1 Timeline for the antibiotics introduction in different era (adapted from reference (10)**)**

Nine most important antibiotic classes are beta-lactams (penicillin), tetracycline, macrolides (erythromycin), glycopeptides (vancomycin), aminoglycosides (gentamicin), lincosamides (clindamycin), sulfonamides (sulfamethizole), oxazolidinones (linezolid) and fluoroquinolones (ciprofloxacin)(Figure 1-2). Among them three classes, sulfonamides, fluoroquinolones, and oxazolidinones, entirely formed from synthetic chemistry. The semi-synthetic derivatives of penicillin formed beta-lactams as an entire class of antibiotics (4). In spite of drug developments and new generations of known drugs, no truly novel drug entered the clinic from 1960s to 2000s.

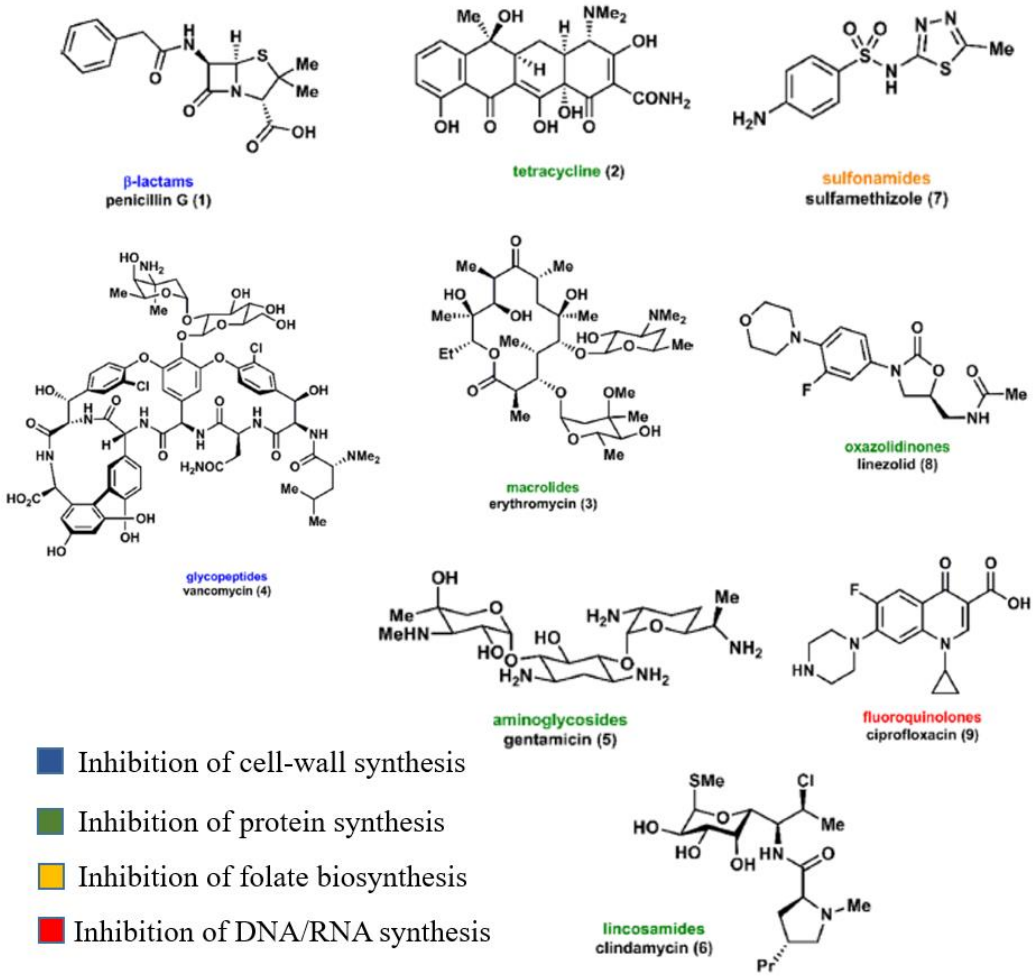


Figure 1-2 Nine classes of antibiotics (11) The mechanism of their action showed by color codes.

The effort of investing in high-throughput screening (HTS) programmes to find new synthetic antibiotics were unsuccessful. For instance among 500 000 compounds screened by GlaxoSmithKline (GSK) using HTS, no candidates were chosen for development (11). For this reason, the old interest of scientists for natural products came up (10).

Most current antibiotics derived from natural products and their main mechanism of actions are inhibition of protein, cell wall and DNA/RNA synthesis (Figure 1-3) (12).

The bacteria can require their resistance through two paths:

1) Biochemically: That caused by antibiotic inactivation (redox process, hydrolysis), target modification (protein structure interference, peptidoglycan structure alteration) or pumps permeability changes.

2) Genetically: That caused by chromosomal mutations (spontaneous, adaptive) or horizontal gene transfer including transduction (bacteriophage), conjugation (plasmids or transposons) or transformation (incorporation into the chromosome of chromosomal DNA or plasmids)(2).

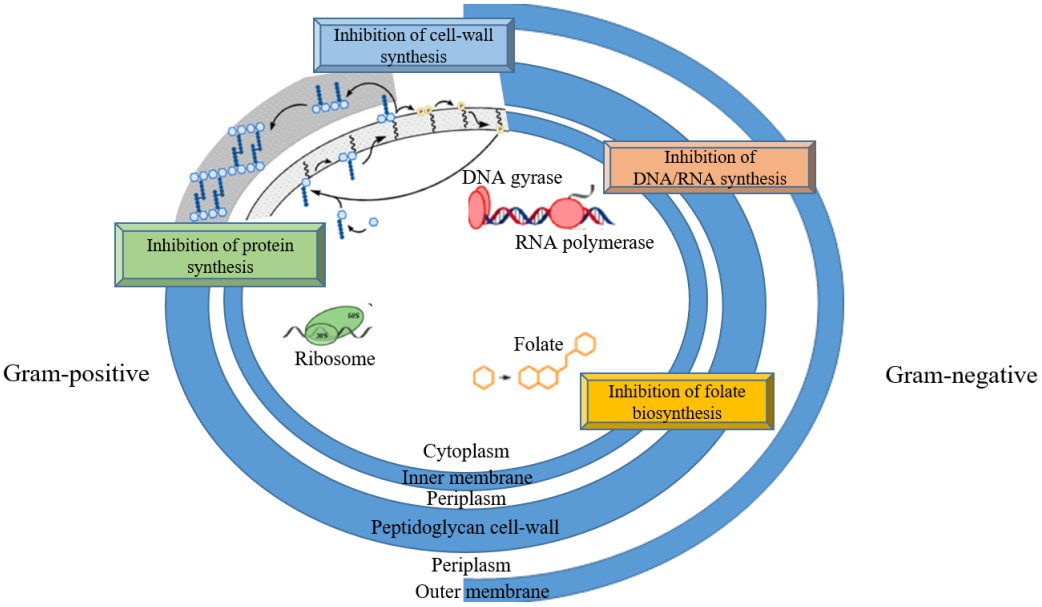


Figure 1-3 Schematic representation of the three major mechanisms of action of widely used antibiotics (12)

Natural products are chemical compounds isolated from different alive creatures and can be primary or secondary metabolites. Primary metabolites are necessary for bacterial growth, development and reproduction and include proteins, nucleic, polysaccharides, fatty acids, etc.)(13). Secondary metabolites, which produce in stationary phase of growth, are often species specific and are not directly essential for growth but involve in multiple functions such as protection (to kill competitors) or communication (signaling molecules between species or mediate interaction with eukaryotic hosts)(11). They can act as dyes/colorants, toxins, hormones, vitamins, cofactors, pharmacologically active agents and antibiotics (14).

1.2 Dereplication

A rising problem with isolating natural products was undesirable duplications for instance, echinomycin, toyocamycin, etc. were discovered independently over 8 to 10 times and named differently (13). That brings an essential element as screening

strategy, to detect secondary metabolite as a new metabolite or as an identical one with what previously published (13). This strategy named dereplication, which was developed by scientists to speed up the natural product discovery, mostly from 1993 until 2014 (15). Dereplication simply means discarding of known compounds (15). It is an approach combining chromatographic or spectroscopic methods with internal and external databases to allow us to identify new secondary metabolites (16). Chapman & Hall's Dictionary of Natural Products (DNP), AntiBase, MarinLit and Myxobase are a number of examples for databases or libraries containing thousands of studied natural products (16). More than two thirds of publications between 2005 and 2015, incorporated the dereplication subject (15).

Is there any limit in finding new microbial compounds? The sources for natural products in the microbial world are significantly unexplored (13).

1.3 Good producers of natural products

Most focus in natural-product discovery has been on the actinomycetes, as they produce 45% of all bioactive microbial metabolites (13). But there are also some Gram-negative and Gram-positive representatives that are good antibiotic producers such as: *Pseudomonas* and *Bacillus* species (13). Recently, Myxobacteria from the phylum Proteobacteria have become one of the top producers of natural products (17).

1.3.1 Myxobacteria

The first myxobacterium was discovered in 1809 and initially introduced as fungus named *Polyangium vitellinum* by H.F. Link (18). Eighty-three years after Link's discovery in 1892, Ronald Thaxter described different species and myxobacterium identified as bacteria. Thaxter's accurate description of *Chondromyces crocatus* and other relatives' unicellular vegetative stage, locomotion, swarming, aggregation and fruiting body formation validated for the next 100 years (19).

Myxobacteria are Gram-negative and δ-proteobacteria belonging to the order Myxococcales. Previously, the order of Myxococcales consisted of 55 species including 28 genera (20) (Figure 1-4), but until 2018, these numbers increased to 30 genera and 62 species (21).

They have rod-shaped vegetative cells and three famous capabilities:

- Their ability to glide and make film-like swarms spread over solid surfaces.
- Their social life and sophisticated intercellular communication systems that bring them all together in large population in their habitats, e.g. soil, wood, dung, etc.
- Their unique morphogenetic potential in starvation conditions, which lead to form a multicellular, species-specific 'Fruiting-bodies' (22).

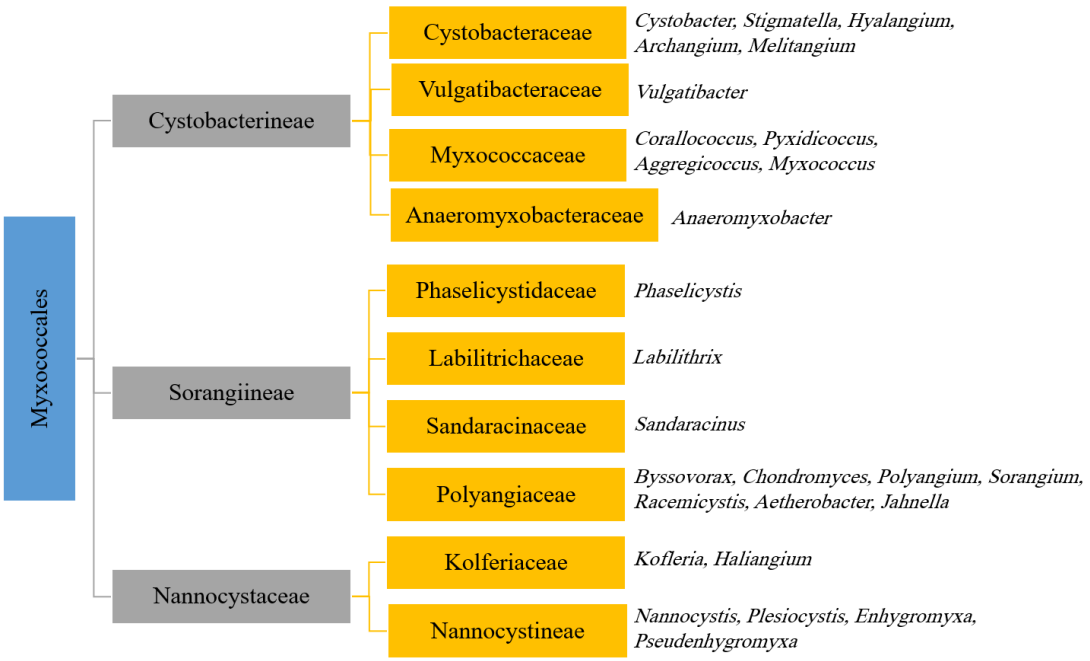


Figure 1-4 Current taxonomy of the order Myxococcales (adopted from reference 20)

Myxobacteria can be divided in two groups of predators and cellulose decomposers. For isolating predators, living *E. coli* is used as the only source of C and N and for the decomposers, filter paper is used. Degradation of large molecules like cellulose or microbial cells require sufficient and large population that can be seen in myxobacteria and let them leave behind many competitors (23).

Other interesting features of them including their large genome, which range in size from 9.0 Mbp to 16.0 Mbp (24) and their extraordinary ability of producing diverse bioactive secondary metabolites (25). Until 2018, from 9000 strains of myxobacteria, more than 100 new carbon skeleton secondary metabolites and 600 derivatives have

been isolated (26). *Sorangium*, *Myxococcus* and *Chondromyces* are the genera with the most isolated natural products (25). The first antibiotic discovery of myxobacteria with antifungal activity was in 1977. Ambruticin was isolated from a strain of *Sorangium cellulosum* (*Polyangium cellulosum)* by Connor *et al*. (20). Clinically used natural products isolated from the same species (*Sorangium cellulosum* So ce90) are Epothilone A and B, which are distinguished by their antifungal and cytotoxic activity but nowadays use for cancer treatment (27).

1.3.2 Actinobacteria

One of the largest taxonomic bacterial phylum, which inhabitants of soil, aquatic environments, plant symbionts, animal pathogens and human or animal gastrointestinal commensals, is the phylum Actinobacteria (28). These Gram-positive bacteria have diverse morphological, physiological and chemotaxonomic properties. The phylum Actinobacteria consists of 6 classes including Actinobacteria, Acidimicrobiia, Coriobacteriia, Nitriliruptoria, Rubrobacteria, and Thermoleophilia, 5 subclasses, 6 orders, and 14 suborders. The order Actinomycetales belongs to the class Actinobacteria and constitute 43 families (29). Initially they were considered as fungi and their name derives from the Greek words of ray (aktin) and fungi (mukes). This phylum involved two main groups defined as Streptomycetes and non-Streptomycetes (30). Actinobacteria have a range of life cycles but the best-studied of them was in *Streptomyces coelicolor* A3 (28). The *Streptomyces* life cycle begins with spore germination and grow until they form substrate mycelium. Due to stress or lack of nutrition, they will form aerial hyphae, which undergo cell division to form spores (Figure 1-5)(31).

- Substrate mycelium: Known also as primary or vegetative mycelium is absorbing nutrients from medium (by getting in to medium or on the surface).
- Aerial mycelium: The hyphae that substrate mycelium develops upright on the surface of the medium mainly to produce reproductive spores.
- Spores: Spores may be formed on the substrate or aerial mycelia as single cells, chain of spores or in vesicles (sporangia) in limited nutrition condition to rescue the bacteria (28,30).

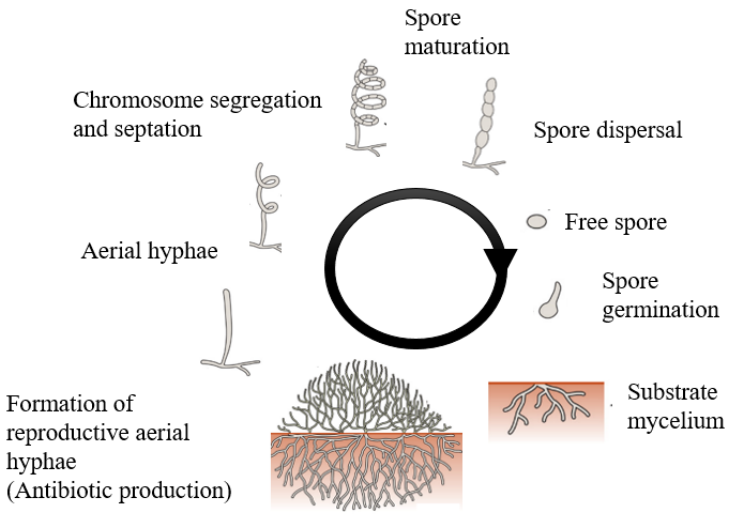


Figure 1-5 Life cycle of Actinobacteria (28)

The nomenclatural and classification of the phylum Actinobacteira were very challenging (29). The earliest genus described by Cohn in 1875 was *Strepthrotrix* (29). Until 1920s naming in taxa or species of this phylum was a major issue. The names were assigned between fungi and bacteria. In 1950 Waksman listed 31 synonyms of names for actinomycetes (29). The lack of an international agreement for classification, made a confusion in establishing new actinomycetes. The confusion solved partly in the eighth edition of Bergey's Manual of Determinative Bacteriology. This is the standard reference for bacterial taxonomy (29). Afterwards the establishment of Approved Lists of Bacterial Names by Skerman in 1980 made a turning point in bacterial taxonomy. Based on that, just the validly described taxa by an international committee of microbiologists were recognized and added to the list. However after 1980, the taxonomic rearrangements at the genus and family level started, due to chemotaxonomic analysis or 16S rRNA sequencing, identification became challenging (29).

1.4 Polyphasic taxonomy

Microbial taxonomy or microbial systematics relies on three main elements: characterization classification and nomenclature (32). The term nomenclature defines as choosing name or labeling in a particular specialist field, which depends on classification. The information, which gathered from characterization is needed for classification (32). The polyphasic taxonomy approach currently used in the

characterization of a novel bacterium includes a combination of phenotypic, chemotaxonomic and genotypic data (33).

Initially, classification was based on phenotypic markers such as morphology or growth conditions. Later, biochemical and physiological aspects were added and until 1980s, the development of sequencing techniques (in particular 16S rRNA gene amplification) facilitated bacterial classification (34). Although from mid-1990s, whole-genome sequencing caused a revolution in taxonomy, but still routine identification of bacterial strains is needed to fulfil the requirements in assigning a novel taxon (34).

For establishing reliable identification of new species, especially in the phylum Actinobacteria (with 29 orders and 62 families), the polyphasic approach is a crucial step.

1.4.1 Phenotype

Phenotype is the observable expression of genotype and it includes morphological, biochemical and physiological properties of the organism (33).

1.4.1.1 Morphology

The phylum Actinobacteria has the richest morphological differentiation (30). The methods applied for analysis of morphology, including colony and pigment formation, aerial and substrate mycelium observation, are conducted by culturing the Actinobacteria on ISP plates (International Streptomyces Project media). Complicated structures such as spore, spore chain and sporangia can be observed by scanning electron microscopy (SEM). Cellular morphology can vary from coccoid (*Micrococcus*) to highly branched mycelia (*Streptomyces*) or elongated filaments (*Rhodococcus*)(28). Spore formation is important in the taxonomy of Actinobacteria, they can be formed as single cells or chains on the substrate and/or aerial mycelium or in special vesicles (sporangia). As examples, the genera *Streptomyces*, *Kitasatospora* and some *Nocardia* spp. have long chains of spores, the genera *Actinomadura*, *Saccharopolyspora* and *Nocardia* spp. produce short chains of spores. *Frankia* and *Kibdelosporangium* keep their spores in sporangia, which looks like bags (26).

Melanoid pigments are also important in taxonomic studies. These metabolic polymers are not essential for growth but their presence improve bacterial survival.

1.4.1.2 Biochemistry

- Chemotaxonomy

Chemotaxonomical characteristics relates to distribution of chemical markers of the cell envelope. The amino acids in cell-wall, sugars in whole-cell and lipids, menaquinones and fatty acid in plasma membrane (26).

Amino acids: Identification of different amino acid sequences of the tetrapeptide side chain of peptidoglycan, the mode of cross-links between the chains like the presence or absence of glycine in interpeptide bridges or the presence of diaminoacids at position 3 of the peptide chain like 2,6-diaminopimelic acid (DAP), give important information for classification of Actinobacteria (28,35,36).

Sugar: The actinobacterial species divided in 5 groups based on the presence of different sugars in their whole-cell (26,33):

A) Group A contains arabinose and galactose
B) Group B madurose (3-O-methyl-D-galactose)
C) Group C no diagnostic sugars
D) Group D arabinose and xylose
E) Group E galactose and rhamnose

Phospholipids: Based on major phospholipid markers in plasma membrane, Actinobacteria have been classified to five groups (33).

Table 1-1 Phospholipid types

Phospholipid types	Characteristic phospholipids
PI	No nitrogenous phospholipids
PII	Only one nitrogenous phospholipid phosphatidylethanolamine
PIII	Phosphatidyl choline and characteristic phospholipid
PIV	Glucosamine-containing phospholipids
PV	Phosphatidylglcerol and glucosamine-containing phospholipids

Menaquinone: Among detected respiratory isoprenoid quinones, which play an important role in the electron transport chain, menaquinones are most commonly found

in actinomycete cell envelope. the length of isoprenoid side chain, and the number of saturated isoprenoid units are important in classification (36).

Fatty acids: Fatty acids are found in cytoplasmic membrane of bacteria, lipoteichoic acids of Gram-positive and lipopolysaccharides of Gram-negative bacteria. Carbon chain length, presence of methyl groups, saturated and unsaturated fatty acids, cyclopropane or hydroxyl fatty acids are important in taxonomic classification (37).

- Enzymology

There are some enzyme tests that are helpful in polyphasic taxonomy. For instance respiratory enzyme tests (oxidase), catalase or hydrolysis of polymers (cellulose, gelatin, starch, etc.).

1.4.1.3 Physiology

Utilisation of different carbon sources, optimal temperature and pH value, resistance to sodium chloride are helpful factors in differentiating between all species of Actinobacteria.

1.4.2 Genotype

The genotype is as important as phenotype in description of bacteria. 16S rRNA gene sequencing, DNA-DNA Hybridization (DDH), Average Nucleotide Identity (ANI), G+C content and whole-genome sequencing are the current methods for identifying bacterial species (37). DNA–DNA hybridization techniques (Brenner et al., 1969; Johnson, 1991) and chemotaxonomy were used for bacterial taxonomy between 1960s and the 1980s. Later, DNA amplification and sequencing techniques (specifically 16S rRNA gene sequencing) facilitated bacterial classification (34). In the mid-1990s, whole-genome sequencing provided complete genome information of the strain. But still identification of a bacterial strain is depending on comparing phenotypic and genotypic characteristics of new strain with previously described species (34).

1.4.2.1 16S rRNA gene

The 16S rRNA gene (1650 bp) is one of the three kinds of bacterial ribosomal RNA molecules (5S, 16S, 23S), which has highly conserved nature and is used as marker for microbial taxonomy. 16S rRNA gene sequences of related species can be retrieved from databases like Ribosomal Database Project II (http://rdp.cme.msu.edu/) and GenBank (http://www.ncbi.nlm.nih.gov/). Phylogenetic analysis can be performed after

alignment of 16S rRNA gene sequence using software packages such as BLAST and CLUSTAL X (33). Phylogenetic tree constructional is using software such as PAUP, PHYLIP, MEGA 4 or the DSMZ phylogenomic pipeline of the Genome to Genome Distance Calculator (GGDC) server available at http://ggdc.dsmz.de/ (38). Stackebrandt and Goebel recommended a 97 % 16S rRNA gene sequence similarity threshold for same species in 1994, whereas today the cut-off identity value for new species is 98.7% (39). Step-by step procedure for taxonomical characterization of newly isolated strains, deposition in culture collection centers and publication is shown in Figure 1-6.

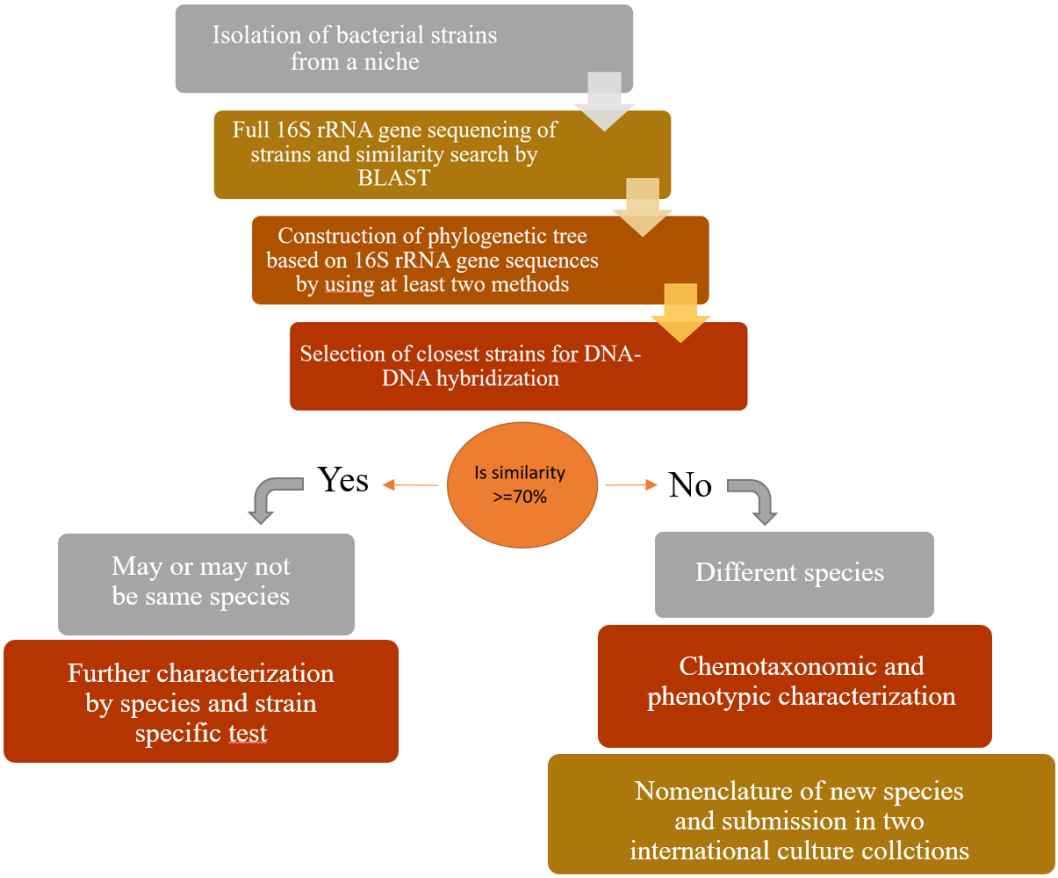


Figure 1-6 Flow diagram of procedure for taxonomical characterization of newly isolated strains adopted from (33)

1.4.2.2 DNA-DNA hybridization (DDH)

DNA-DNA reassociation or hybridization is a technique based on denaturation of DNA from two bacterial species in high temperature and then making a hybrid by lowering

down the temperature. Bacterial species are detected as new species when their DDH value is ≤ 70%.

DDH value is depends on three parameters (33):

1) G+C mol %
2) Ionic strength of solution
3) Melting temperature of DNA hybrid

Nowadays digital DNA-DNA Hybridizations (dDDH) have more advantages in comparison to experimental DDH, because the data are accessible and accumulative on available databases (40).

1.4.2.3 Average Nucleotide Identity (ANI)

This is a valid alternative to DDH, introduced by Konstantinidis and colleagues (33). It is a tool, which gives the mean nucleotide sequence identity of shared genes between two strains. The ANI value of 95% corresponds to DDH value of 70%. This tool should not be used as a single tool for classification, although there are some examples of novel bacteria descriptions with ANI such as *Dehalococcoides mccartyi* (Loffler *et al*., 2012) and *Sphaerochaeta globose* (Ritalahti *et al*., 2012)(34).

1.4.2.4 G+C content

The analysis of guanosine and cytosine (mole percent) of DNA is important in bacterial systematic. Because the variation of GC content percentage is not more than 3% within a well-defined species and not more than 10% within well-defined genera (33).

1.4.2.5 Whole-genome sequencing

Over the past few years, Next Generation Sequencing Technologies (NGST) are widely used for accurate sequencing of bacterial genomes (41). Previously, there was no need to perform whole-genome sequencing for novel species description. Whereas recently, the International Journal of Systematic and Evolutionary Microbiology (IJSEM), which is famous for publishing new strains description, made it mandatory to do whole-genome sequencing (http://ijs.microbiologyresearch.org/content/Genome_data_required_IJSEM. Accessed 22 April 2019) (42). One of the databases for genome-based prokaryote taxonomy, which grows continuously is the Type (Strain) Genome Server (TYGS) (https://tygs.dsmz.de/)(42). This database contains comprehensive information of the genomes of type strains of

species and subspecies with validly published names, automatically detection of closest neighbors of query genomes and truly whole-genome-based methods for phylogeny and classification (40).

1.4.2.6 MALDI-TOF

Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) MS spectra give some information about ribosomal proteins, which can be used in taxonomy of bacteria (43).

1.4.3 Ecology

Actinomycetes are ubiquitous and can be found in soil, freshwater and marine environments. Beside their interaction with environment, they are welcome guests in many other organisms like plants (*Streptomyces* as endosymbionts and pathogens), insects (form mutualistic symbiosis in beewolves, fungus growing ants), marine animals (in the microbiome of sponges, sea-cucumbers, seaweeds, cone snails) and etc (44). The phylum Actinobacteria is famous for producing antibiotics and in most mutualistic symbiosis interactions (45), they have a protective role (46). On the other hand, they produce enzymes that degrade resilient biopolymers (42). The most important genus in the phylum Actinobacteria is the genus *Streptomyces* (28). Some examples of the biological functions of the interactions between *Streptomyces* spp. and their host organisms are shown in Figure 1-7.

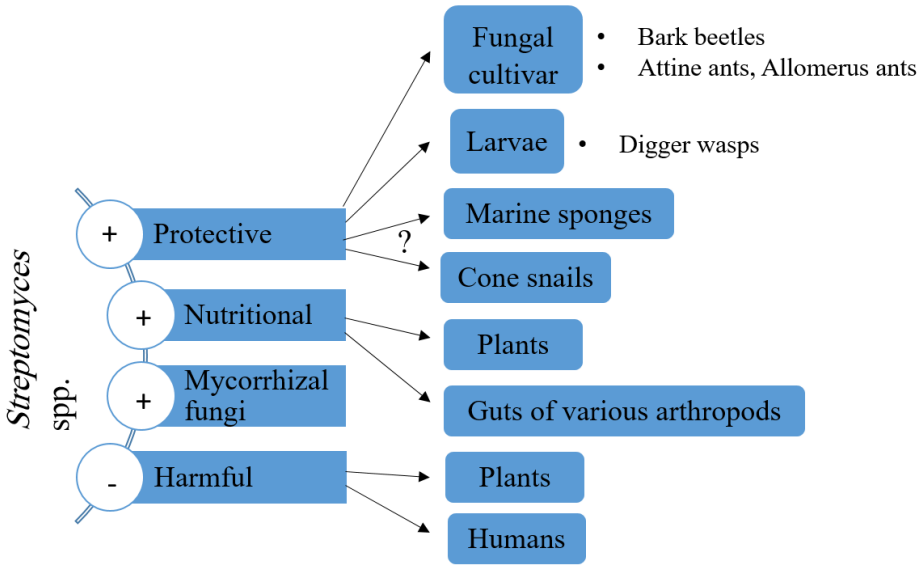


Figure 1-7 Some examples of interactions of Streptomyces spp. with eukaryotic organisms (28)

The ecology or specifically the nutrient status of the environment, has a key role in producing antibiotics by actinomycetes (44). So it brings the idea of finding known species that can produce novel secondary metabolites because of their uncommon habitats or apply different isolation methods for focusing on under-investigated species.

1.4.3.1 Uncommon habitats

The reduction in discovery of new antibiotics and the rise of rediscovery of known strains specially from terrestrial environments, made scientists to think about new sources. However, terrestrial microorganisms are still being discovered as novel natural product producers. Screening uncommon habitats like mangrove forests, deserts, seas etc. may result in discovery of new strains that are more likely to produce new natural products (13).

- Medicinal plants

Many studies were conducted on Actinobacteria in medicinal plants (47). The use of herbal remedies is deeply tied up in culture and medicine especially in Asia (47). Many medicinal plants-associated Actinobacteria are able to inhibit or kill pathogenic bacteria, fungi or viruses (48). Some novel bioactive compounds isolated from plant-associated Actinobaceria are shown in Table 1-2.

Table 1-2 Bioactive compounds isolated from plant-associated Actinobacteria

Actinobacteria	Plant	Bioactive compounds	Type	Reference
Streptomyces sp. NRRL 30562	*Kennedia nigriscans*	Munumbicins A-D	Peptides	(49)
Streptomyces sp. NRRL 30566	*Grevillea pteridifolia*	Kakadumycins	Peptides	(50)
Streptomyces sp. CS	*Maytenus hookeri*	24-demethyl-bafilomycin C1	Macrolides	(51)
Streptomyces albidoflavus	*Bruguiera gymnorrhiza*	Antimycin A18	Macrolides	(52)
Micromonospora lupini	*Lupinus angustifolius*	Lupinacidins A and B	Anthraquinones	(53)

- Mangrove forests

Mangroves are defined as tropical trees, which grow between the high spring tide and near mean sea levels. They cover almost 75% of the world's tropical and subtropical

coastlines (54). There have been many Actinobacteria isolated from mangrove forests but among them five different genera appeared to be valuable sources of potentially useful bioactive metabolites (53). Some novel bioactive compounds isolated from mangrove forest's Actinobaceria are shown in Table 1-3.

Table 1-3 Bioactive compounds isolated from mangrove forest's Actinobacteria

Actinobacteria	Place of mangrove forest	Bioactive compounds	Activity against	Reference
Micromonospora rifamycinica AM105	Hainan Island, South China Sea	rifamycin S	*Staphylococcus aereus* (MRSA)	(55)
Jishengella endophytica 161111	Hainan Province, China	β-carboline	H1N1 virus	(56)(57)
Salinispora tropica CNB-392	Chub Cay, Bahamas	salinosporamide A	cancer cells	(58)
Saccharopolyspora sp. RL78	Nosoko, Ishigaki Island, Japan	JBIR-102	cancer cells	(59)
Nocardiopsis sp. A00203	Jimei, Fujian Province, China	nocardiatones A	cancer cells	(60)

- Deserts

Although deserts cover one third of earth's surface, little data are available from new chemical compounds produce by Actinobacteria. Because it was believed that such conditions like scarcity of water, made life impossible (61). On the other hand, scientists thought that there would be no competition that microorganisms were likely to synthesize bioactive metabolites (62). But from 2010, after investigating novel Actinobacteria isolated from Atacama Desert soil, this belief has been changed (61). Some novel bioactive compounds isolated from desert's Actinobaceria are shown in Table 1-4.

Table 1-4 Bioactive compounds isolated from Actinobacteria of desert

Actinobacteria	Desert	Bioactive compounds	Bioactivity	Reference
Streptomyces sp. DB634	Atacama, Chile	abenquines A–D	Antibacterial, antifungal	(63)
Streptomyces leeuwenhoekii C34^{T}	Atacama, Chile	chaxamycins A–D	Antibacterial	(64)
Saccharothrixs SA198	Saharan, Algeria	antibiotic A4	Antibacterial and antifungal	(65)
Kibdelosporangium aridum subsp. largum	Pima County, Arizona, USA	kibdelin	Antibacterial	(66)

- Freshwater snails

The interaction of Actinobacteria with vertebrates and invertebrates have been studied in different articles. Actinobacteria mostly influence their host's ecology by different functions like producing different enzymes for digestion or protecting the host from other pathogens (28). As an example European beewolf that harbors the antibiotic-producing "Candidatus *Streptomyces philanthi*" in antennal glands to protect wasp larvae from fungal infections (67). The mutualistic *Streptomyces* incorporated into beewolf cocoons provide a combination of nine antibiotic substances, including streptochlorin and a complex of eight piericidin derivatives, which are expected to protect the host against the fungal pathogen. In return, the host provides nutrition and a competition-free ecological niche for the bacterium (45).

Invertebrates are a diverse group of animals that have interaction with useful microbes such as Actinobacteria in different ecosystems (68). Although previously insects and nowadays marine invertebrates, received considerable attention for their natural products but some sources like freshwater snails should not be under-investigated (69,70). Freshwater snails are important because they act as intermediate host for over 10,000 species of trematodes (71). It is believed that the freshwater snails microbiota (especially Actinobacteria) can prevent the invasion of pathogenic microbes (70). Snail-associated Actinobacteria were investigated by Peraud *et al*. in 2009 for the first

time (72). *Streptomyces* sp. CP32 was isolated as a new strain from the cone snail *Conus pulicarius* and produces the neuroactive thiazoline metabolites pulicatins A-E (73).

To our knowledge there are no studies on secondary metabolites produced by freshwater snail-associated Actinobacteria.

- *Physa acuta*: It is a globally invasive freshwater snail native to North America (74). This small, left-handed, air breathing genus has widespread distribution in all over the world and acts as a host for trematodes, causing diseases like echinostomiasis and fascioliasis (75).

1.4.3.2 Selective isolation methods

It is well-known that only 1% of bacteria on the earth have been cultured. The majority of bacteria remain unexplored, mostly due to standard cultivation methods (76). Various cultivation methods can lead in the isolation of different groups of bacteria (77). Each group of bacteria requires specific growth conditions including different temperatures, pH, oxygen level and nutrients. When the artificial medium or incubation conditions are different from bacterial requirements, the bacteria will not grow. In some cases, the bacteriocins released from other bacteria in a mixed culture inhibit the bacterial growth (76).

Actinobacteria can be divided into two groups:

Streptomyces

Non-*Streptomyces* (Rare Actinobacteria)

The best strategies to reach a specific group of bacteria (like rare Actinobacteria) are (78):

- Pretreatment methods

  Drying and heating

  Adding chemicals such as phenol and chloramine-T

  Adding antibiotics like leucomycin and tobramycin

- Design of improved selective isolation media

  HV agar: Humic Acid-Vitamin Agar contain humic acid as the sole carbon and nitrogen source. Actinomycetes can utilise humic acid to support sporulation (79). For eliminating other fast growing bacteria, antibiotics like nalidixic acid can be added (78).

Applying multiple cultivation methods at the same time will robust the chances of isolating rare Actinobacteria (76).

➢ Rare Actinobacteria

Rare Actinobacteria referred to strains that are difficult to isolate under normal cultural conditions. Currently, the discoveries of new natural metabolites are focusing on non-*Streptomyces* or rare Actinobacteria (80). The *Streptomyces* species produce 7600 compounds, while rare Actinobateria produce 2500 (13). The compounds produced by rare Actinobacteria are diverse, unique, complicated and mostly show low toxicity and high antibacterial activity (13), for instance, rifamycins, erythromycin, vancomycin, gentamicin are produced by *Amycolatopsis mediterranei*, *Saccharopolyspora erythraea*, *Amycolatopsis orientalis* and *Micromonopsora purpurea* respectively. Figure 1-8 shows the relative distribution of the bioactive compounds produced by rare actinomycetes, which are described by The Advanced Biological Laboratories (ABL) Database (81).

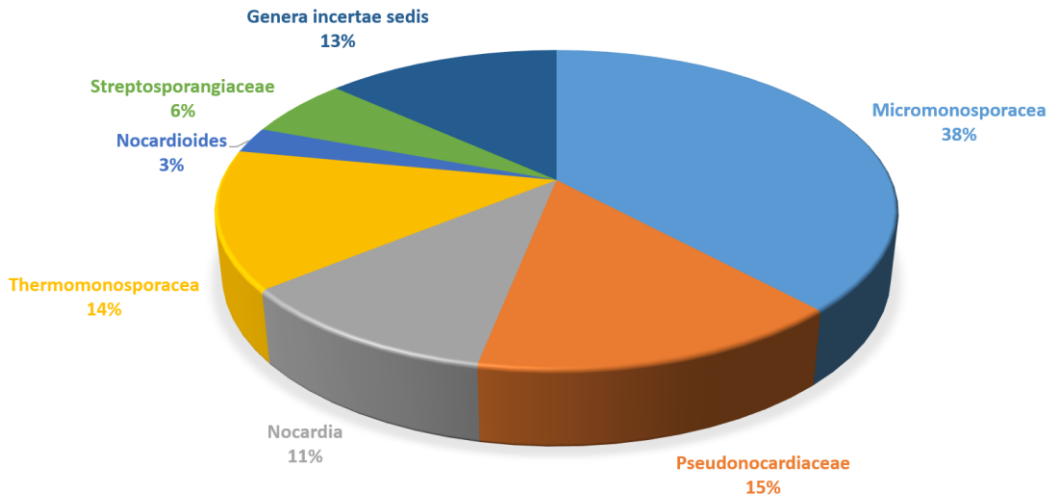


Figure 1-8 Relative distribution of producing strains among rare Actinobacteria adopted from (81)

1.5 Aim of study

The aims of this study step by step are concluded as:

- Isolation of Actinobacteria and Myxobacteria from soil samples collected from uncommon habitats of Iran including desert, mangrove forest, medicinal plants and from freshwater snails collected in Germany
- Purification, identification and phylogenetic analysis of isolates
- Evaluation of antimicrobial activity based on Minimum Inhibitory Concentration (MIC)
- Study the microbiome of one chosen freshwater snail
- Dereplication (Chemical analysis of crude extracts)
- Isolation of the potentially new secondary metabolite from novel actinobacterial strain
- Polyphasic taxonomy and characterization of novel strains
- Genome sequencing of some isolates using TYGS

2 Material and methods

2.1 Media

All of the used media in this study were listed in Supplementary.

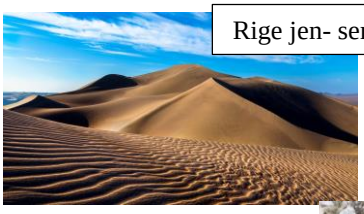
2.2 Sampling date and locations

2.2.1 Soil

Actinobacteria and Myxobacteria were isolated from soil samples collected in different cities (Table 2-1) in Iran (in January 2018) and Germany (in August 2019). The samples were collected from uncommon habitats like local medicinal plants root (Cities: Yasooj, Tafresh), beach sand (Cities: Boushehr, Qeshm) and desert (Cities: Semnan, Kerman, Isfahan) (Figure 2-1).

Table 2-1 List of collected samples with date and country

Date of isolation	Country	City	Type of soil
Feb-18	Iran	Yasooj	Plant soil
		Boushehr	Beach sand
		Tafresh	Plant soil
		Qeshm	Beach sand
		Semnan	Desert sand
		Isfahan	Soil
Jul-18		Kerman	Desert sand
Mar-19		Saravan	Soil
		Lorestan	Soil
Jul-19		Qeshm	Beach sand
Aug-19	Germany	Braunschweig	Plant soil
Oct-19	Iran	Isfahan	Soil



Rige jen- semnan desert

Astragalus - Yasooj

Silver island- Qeshm

Figure 2-1 The different sampling sites in Iran (red dots)

2.2.2 Snail

Different genera of freshwater snails (Figure 2-2) were collected during April 2017 to August 2019 from six different regions in Iran and Germany (Table 2-2).

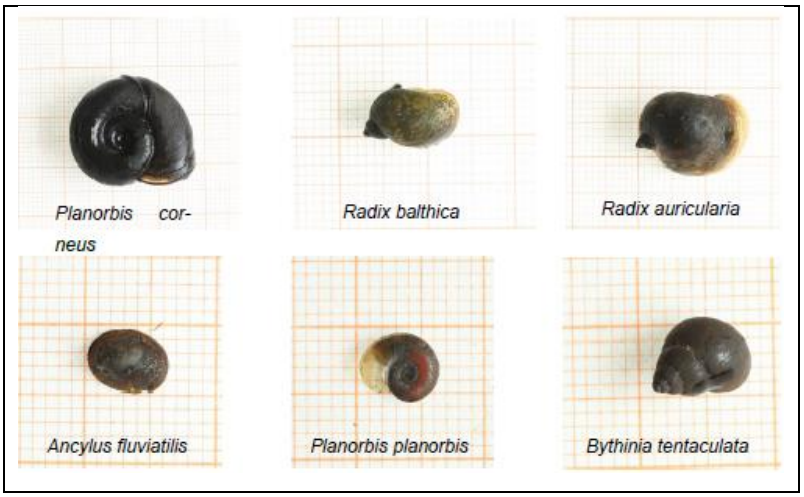


Figure 2-2 Some collected freshwater snails

Table 2-2 List of collected freshwater snails with date and the country of collection

Date of sampling	Country	City	Genus of collected snails
April 2017	Iran (North)	Mahmodabad (Rice field)	*Lymnaea, Physa, Succinea, Planorbis, Radix, Thiaridae*
		Chamestan (Forest)	
		Chalous (Side road)	
September 2017	Germany (Lower Saxony)	Schladen	*Planorbis, Lymnaea, Bithynia*
		Isingerode (Fishing area)	
July 2018	Germany (Hessen)	Hanau	*Succinea, Planorbis, Radix, Lymnaea, Potamopyrgus*
		Gießen	
		Walluf	
		Darmstadt	
		Rödermark	
		Darmstadt (Botanischer Garten)	
		Gießen	
		Rödermark	
		Gießen	
May 2019	Germany (Lower Saxony)	Braunschweig	*Bithynia, Ancylus, Physa, Planorbis, Potamopyrgus, Lymnaea*
June 2019			
August 2019			

2.3 Isolation and purification

2.3.1 Myxobacteria

Myxobacteria grow slowly compared to most soil bacteria. Their swarming behavior facilitates the isolation process. To isolate predator Myxobacteria, water-agar plates with living *E. coli* as the only C and N-source were used. Living *E. coli* must be streaked on water-agar plates like a cross and at the end of the cross, soil or snail samples can be added. To isolate cellulose degrading Myxobacteria like *Sorangium* and *Byssovorax,* mineral salt agar plates (Stan 21=ST21) with autoclaved filter paper in the center were used (Figure 2-3). The plates were incubated at 30 ˚C for three weeks. Swarms and fruiting bodies from water agar and ST21 plates transferred with needle to new plates. After several transferring steps, the purification continues on VY/2 plates (Appendix). Myxobacterial vegetative cells should be checked under a microscope. The vegetative cells are rod shaped without moves (82).

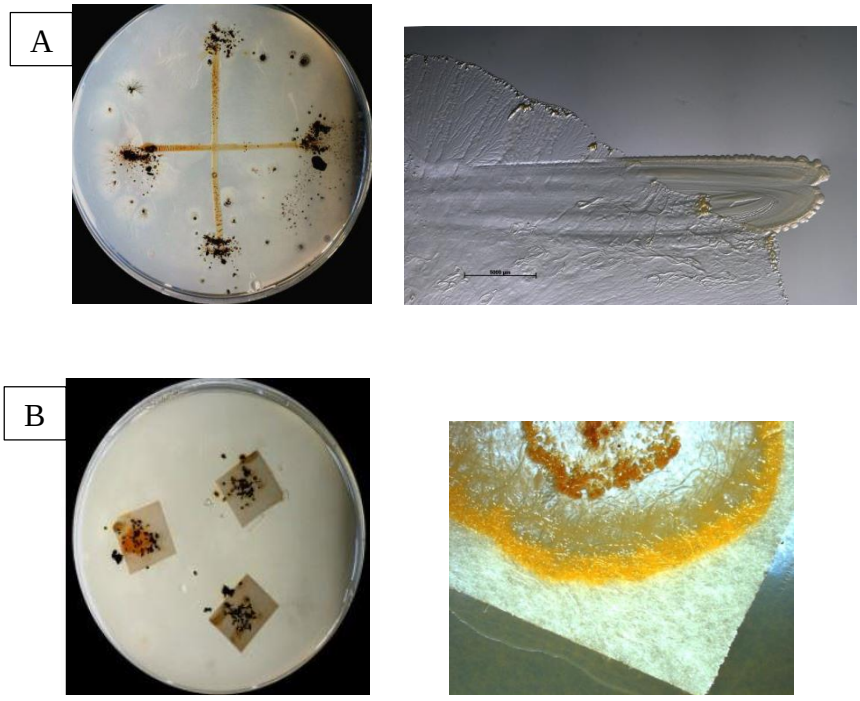


Figure 2-3 Isolation of predators (A) and cellulose decomposers (B)

2.3.2 Actinobacteria

To isolate Actinobacteria, 1 gram of soil was mixed with 10 ml of sterile water in a 15 ml falcon tube and vortexed. Serial dilution was conducted as follows: 1 ml of the stock solution mixed in 9 ml sterile water to get a 1:100 and repeated to get a 1:1000 dilution.

The routine procedure for isolation was to plate 100 µl of each dilution on medium 5336 agar supplemented with 1% cycloheximide to reduce fungi contamination. The plates incubated for 7 to 21 days at 30 ˚C. The purification continued using GYM medium (78).

One of the soil samples from a desert in Isfahan, Iran was targeted to isolate specifically rare Actinobacteria. For this reason, pretreatment was performed by heating the soil at 60 ˚C for 30 minutes and adding chloramine T (83). Instead of 5336 medium as was used for the first isolation step in the routine procedure, Humic acid-vitamin agar (HV), ISP4 and ISP2 plates were used (84).

2.3.3 Myxobacteria and Actinobacteria from snails

To isolate Myxobacteria and Actinobacteria from freshwater snails, the process differed based on the snail size. For instance, the freshwater snail *Lymnaea* (because of its big size) needed to be boiled in water for a minute to take it out of its shell before it could be dissected. For all other genera used in this study, boiling was not necessary. The whole snails were sterilized for 30 seconds in 70% ethanol. Then they were transferred to 1.5 ml Eppendorf filled with 500 µl sterile water and crashed by sterile pestle. Afterwards from this homogenized sample, 200 µl applied on 5336 agar medium, water agar and ST21 agar.

2.4 Identification

- **DNA extraction**

To identify microbial strains, DNA was extracted using the Invisorb® Spin Plant Mini Kit (Stratec Molecular, Germany) through the following process: from GYM liquid for Actinobacteria and from CYH liquid medium for Myxobacteria, 500 µl of medium were transferred to a 1.5 ml Eppendorf. For Actinobacteria, one step of homogenizing with pestle is needed. Samples were centrifuged for 1 min at 11000 rpm. The supernatants were discarded, and 400 µl lysis buffer P and 20 µl proteinase S were added. After vortexing the samples, they were incubated at 65 ˚C for 30 min. Then the lysis solution was transferred to prefilters and centrifuged for 1 min at 11000 rpm. The prefilters were discarded and 200 µl of binding buffer A was added to the samples after which the samples were vortexed. The suspension was transferred to a spin filter, incubated for 1 min and centrifuged at 11000 rpm for 2 min. 550 µl of wash buffer I was added to the filter and centrifuged at 11000 rpm for 1 min. After discarding the filtrate, the spin filter was placed into the 2 ml receiver tube. 550 µl of wash buffer II

was added and centrifuged at 11000 rpm for 1 min for two times. The drying step continued with centrifuging for more than 4 min at 11000 rpm. Then the spin filter was placed into a new 1.5 ml receiver tube and 30 µl of the prewarmed elution buffer added. After 3 min of incubation, the samples centrifuged for 1 min at 11000 rpm. Finally, the extracted DNA was stored at 4 ˚C.

- **PCR amplification**

Amplification of the 16S rRNA gene was achieved with PCR technique and purification of the PCR products was carried out using the NucleoSpin® Gel and a PCR Clean-up kit. Amplification of the 16S rRNA gene was performed using a special master mix JumpStart™ Taq ReadyMix™ (by Sigma-Aldrich). The PCR mixture had a final volume of 50 µl and consisted of the following components: 25 µl JSRM, 22 µl H_2O, 1 µl forward 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1 µl reverse primer 1492r (5'-GGTTACCTTGTTACGACTT-3') with 1 µl of the template DNA (Lane, 1991). The PCR conducted with thermocycler (Eppendorf Thermocycler Mastercycler gradient), started with temperature of 95 ˚C for 5 min, followed by 34 cycles of denaturation (94 ˚C, 30 sec), primer annealing (52 ˚C, 30 sec) and elongation (72 ˚C, 2 min). The final elongation reaction was performed at 72 ˚C for 10 min. Samples were mixed with a loading buffer and charged with the DNA-dye SYBR-Green and then loaded on a 0.8% agarose gel. After running 45 min at 70 V DNA bands were detectible under UV light.

- **Purification of PCR products**

To clean up the isolated PCR products, 1 volume of sample with 2 volumes of buffer NTI were mixed and placed in a nucleospin® gel column, which was placed in a collection tube. After centrifuging for 30 sec. at 11000 rpm, 700 µl of buffer NT3 was added and centrifuged. The nucleospin gel column was placed in a 1.5 ml microcentrifuge tube and 30 µl buffer NE was added. After 1 min of incubating at room temperature, a centrifugation for 1 min at 11000 rpm followed to get the DNA. The DNA sequencing was performed by using the 96-capillary-system from Applied Biosystems (ABI), 3730xl DNA Analyzer. Additional primers 1100f (5'-CAACGAGCGCAACCC-3'), 1100r (5'-GGGTTGCGCTCGTTG-3') and 518r (5'-CGTATTACCGCGGCTGCTGG-3') were used to obtain an almost full-length 16S rRNA gene sequence (1459bp). The acquired sequences were assembled using the

program Bioedit (version 7.2.6) and later the consensus sequences were compared against the EzTaxon Database (85).

- **Phylogenetic analyses**

Calculation of pairwise 16S rRNA gene sequence similarities was performed using the EzTaxon server (https://www. ezbiocloud.net) (86) and the maximum-likelihood (ML) and maximum-parsimony (MP) trees were carried out using the DSMZ phylogenomic pipeline of the Genome to Genome distance calculator server (39) available at http://ggdc.dsmz.de/. Muscle (87), RAxML (88) and TNT (89) were used for performing multiple sequence alignment.

2.5 Screening for secondary metabolites

- **Harvesting the metabolite cultures**

For Actinobacteria, the preculture of GYM liquid was used after 7 days. 1:10 of this culture was transferred to metabolite production media 5254 and 5294. The cultures were incubated at 30 ˚C and 160 rpm on a rotary shaker for 7 days. To get the crude extract of the cultures, 20 ml of the culture was mixed with 20 ml of ethyl acetate and shook for one hour and centrifuged at 9000 rpm for 15 min. The upper phase was transferred in a round-bottom flask and evaporated with a rotary evaporator (Heidolph Laborata 4003). The crude extract was dissolved in 200 µl of methanol.

For Myxobacteria, H-medium was used as a preculture medium. After 2 weeks of incubation at 30 ˚C and 160 rpm, 1:10 of the cultures was transferred in 100 ml of different production media like POL, P and Myxoverisin with 2% XAD adsorber-resin. For screening secondary metabolites from the genus *Myxococcus,* POL, P and Myxoverisin media were used. For genera *Corallococcus*, *Archangium*, *Sorangium*, *Hylangium* and *Nanocystis*, POL, P, Myxoverisin and E medium were used. Cultures were harvested after 10 days of incubation. The cultures were sieved with a 200 µm sieve. XAD was transferred to a flask and filled with 70% acetone and left for one hour. After evaporating, the crude extract was dissolved in methanol.

- **Antimicrobial test assay**

The crude extracts were screened against nine test microorganisms with Minimal Inhibitory Concentration (MIC)(90). Tested bacteria included *Escherichia coli* DSM 1116, *Staphylococcus aureus* (Newman), *Mycobacterium smegmatis* ATCC 700084, *Citrobacter freundii* DSM 30039, *Pseudomonas aeruginosa* PA14, *Bacillus subtilis*

DSM 10^T and tested fungi as *Wickerhamomyces anomala* DSM 6766, *Mucor hiemalis* DSM 2656^T and yeast *Candida albicans* DSM 1665.

The MIC was determined by using the final concentration of 1 mg/ml of crude extract with seven 1:2 serial dilution steps in 96-well microplates. Mueller Hinton Broth medium (MHB: 0.5% casein peptone, 0.5% protease peptone, 0.1% meat extract and 0.1% yeast extract, pH 7.0) was used for bacteria and MYC medium (1.0% glucose, 1.0% phytone peptone and 50-mM HEPES (11.9 g/l) for fungi. 20 µl of crude extract was added to the first row of wells, which contained 280 µl of bacterial/fungal suspension. After mixing, 150 µl was withdrawn and transferred to the next row of wells. This was repeated sequentially up to the last well. A dilution gradient of 66.6, 33.3, 16.6, 8.3, 4.2, 2.1, 1.0 and 0.52 µg/ml was obtained by discarding 150 µl from the last well. All plates were placed on a microplate shaker incubated for 24 h at 650 rpm at 30 °C for all plates except *M. smegmatis* ATCC 700084 and *E. coli DSM* 1116, which were incubated at 37 °C. The MIC values were defined as the lowest concentration of the tested extract where the growth of the tested microorganisms was not visible to the naked eye.

- **Detection of potential compounds**

Later, the crude extracts with antibacterial activity were fractionated using Reversed-Phase HPLC (RP-HPLC) on an Agilent 1100 HPLC system equipped with an XBrigde® (Agilent Technologies, Santa Clara, CA, USA) C-18 3.5µm, 2.1mm × 100mm, Waters column. The fractions (0.15 ml) in the 96-well plates were collected by the HPLC column every 0.5 min and were later dried for 45–60 min at 40 °C using heated nitrogen. Afterwards, each well was filled with 150 µl of the bacteria, which the crude extract showed activity against. Crude extracts were analysed by HR-ESI-MS (high-resolution electrospray ionization-mass spectrometry) using the following setup: An HR-ESI-MS Agilent 1200 system coupled to a DAD (Diode-Array Detection) and a maXis ESI TOF (time of flight) mass spectrometer (Bruker Daltonics, Bremen, Germany), scan range 100–2500 atomic mass unit (amu), rate 2 Hz, capillary voltage 4500 V and drying gas temperature 200 °C using the following HPLC conditions: C18 Acquity UltraPerformance Liquid Chromatography (UPLC) Ethylene Bridged Hybrid (BEH)(Waters) column (2.1 × 50 mm, 1.7 µm), solvent A: H2O + 0.1% formic acid and solvent B: acetonitrile (ACN) + 0.1% formic acid, gradient: 5% B for 0.5 min, increasing

to 100% B in 20 min, maintaining isocratic conditions at 100% B for 10 min, flow = 0.6 ml/min and UV−Vis detection 200−600 nm. Data processing and analysis was done using data analysis software included in the Compass software from Bruker in order to identify the bioactive target masses. The obtained molecular features were matched against known actinobacterial compounds from the Dictionary of Natural Products (DNP: http://dnp.chemnetbase.com/) and myxobacterial compounds from the in-house database (Myxobase)(91).

2.6 Culture-independent study of freshwater snails

- Collection and targetting freshwater snails

Three species of freshwater snails including *Lymnaea baltica, Lymnaea stagnalis* and *Physa acuta* were collected from a pond (52˚ 6′ 42.32″ N, 10˚ 40′ 46.20″ E) in Remlingen-Semmenstedt, Germany. The snails were transferred to the laboratory within two hours and kept at room temperature. After 24 hours, the snails were washed with tap water and sterilized with 70% ethanol to wipe the shells. Snails from the genus *Lymnaea* were taken out of their shells and the snails of genus *Physa* had their shells crashed using a sterile pestle before they were transferred to 1.5 ml Eppendorf tubes with 500 µl sterile water. The homogenized samples were used for DNA extraction.

- DNA extraction and Illumina MiSEQ sequencing and data analysis

The DNA of three snails was extracted using the PowerSoil® DNA Isolation Kit and quantified using a nanodrop (Thermo Scientific)(92). From each snail a part was transferred to an Eppendorf, filled with 500 µl sterilized water and homogenized with pestle. 250 µl was added to a PowerBead Tube and then vortexed. 60 µl of solution C1 was added and the suspension vortexed for 10 min. The tubes were centrifuged at 9000 rpm for 30 sec. Supernatant was transferred to a 2 ml collection tube. 250 µl of solution C2 was added to the tube, which was then incubated for 5 min at 2–8 ˚C. After centrifuging for 1 min, the supernatant was transferred to a new collection tube. 200 µl of solution C3 was added and incubated for 5 min. After centrifuging, 750 µl of supernatant was mixed with 1200 µl of solution C4 and 675 µl of suspension, which was then transferred to a MB spin column and then centrifuged for 1 min. Now 500 µl of solution C5 was added to the spin column and centrifuged for 30 sec. Then the spin was placed in a clean 2 ml collection tube and 100 µl solution C6 was added. After centrifuging for another 30 sec, the spin was discarded, and DNA remained in the collection tube. The extracted DNA was analysed for microbial diversity at the Leibniz

institute DSMZ. For this analysis, amplicons of the V3 region of the 16S rRNA gene were prepared with the primer pair 341f and 515r (93). The quality of the Bartram libraries was checked with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, U.S.) and the subsequent sequencing was performed in 150 bp paired end mode on a NextSeq™ 500 (Illumina®, San Diego, CA, U.S.). The generated sequences were processed with an amplicon analysis pipeline after the quality of the raw reads had been evaluated by FastQC version 0.10.1 (Simon Andrews; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). After trimming the forward and the reverse reads to a length of 130 bp, the raw-sequence data was purified from potential primer dimers by a JAVA program called *DimerFilter*. Fastq-join (94) joined the forward and reverse reads with a 20 percent mismatch and a minimum overlap of 6 bp. FASTA-converted sequence files were subsequently checked with Uchime (95) against the Gold database provided by ChimeraSlayer (Robert Edgar, Drive5, Corte Madera, CA, USA) (http://drive5.com/otupipe/gold.tz) and the RDP classifier 2.10.1 (93). The RDP classifier 2.10.1 with a confidence value of 0.5, which is recommended for short read amplicon data, was employed to perform taxonomic-dependent analyses of the bacterial community (96). V3 amplicon data was deposited at NCBI SRA (Sequence Read Archives) under accession number PRJNA665462. SRA.

2.7 Selection of bacteria

2.7.1 Actinobacteria

16S rRNA gene sequence data provides an initial indication of the taxonomic status of a strain. Considering the low amount of similarities between this specific gene and the bioactivity screening of the crude extracts obtained from them, fifteen candidates, were chosen for further studies. For five strains, whole-genome sequencing and polyphasic approach were performed. These strains are highlighted in red color in Table 2-3.

Table 2-3 List of targeted samples of Actinobacteria

Targeted samples	Similarity 16S rRNA (complete)	
NS40	*Streptomyces badius*	98.55
NS36	*Lentzea violacea*	99.10
NS66	*Amycolatopsis albidoflavus*	99.79
NS37	*Nocardiopsis umidischolae*	99.58
NS38	*Nonomuraea harbinensis*	99.65

NS25	*Actinomadura apis*	99.23
Q1	*Amycolatopsis rifamycinia*	99.03
Q2	*Pseudonocardia cypriaca*	99.65
NS34	*Kribbella pittospori*	99.85
NS59	*Streptomyces specialis*	98
NS44	*Herbidospora sakaeratensis*	97.64
44ZA	*Sanguibacter keddieii*	98.34
BA	*Agromyces persicus*	99.08
Sef	*Prauserella alba*	100
4NS15	*Kibdelosporangium persicum*	98.96

All close phylogenetic relatives of the targeted strains Q1, Q2, 44ZA, BA, 4NS15 were obtained from the German Collection of Microorganisms and Cell cultures (DSMZ) and wink compendium for comparison under the same laboratory condition (Table 2-4).

Table 2-4 Selected strains and their related type strains

Genus	Strain	DSM number	close type strains
Amycolatopsis	Q1	DSM110486	
		DSM46095	*A. rifamycinica*
		SB026	*A. rhabdoformis*
		DSM44652	*A. kentuckyensis*
		DSM44653	*A. lexingtonensis*
		DSM44654	*A. pretoriensis*
		DSM44591	*A. balhimycina*
Pseudonocardia	Q2	DSM110487	
		DSM45511	*P. cypriaca*
		DSM45671	*P. hierapolitana*
		DSM44774	*P. zijingensis*
		DSM45352	*P. adelaidensis*
Sanguibacter	NS44ZA	DSM110740	
		DSM10542	*S. keddieii*
Agromyces	BA	DSM111422	*A. persicus*
		DSM26681	*A. indicus*
Kibdelosporangium	4NS15	DSM110728	*K. persicum*
		DSM43828	*K. aridum* subsp. *Aridum*
		DSM 44150	*K. aridum* subsp. *Largum*
		DSM44226	*K. philippinense*
		DSM104448	*K. phytohabitans*

2.7.1.1 Polyphasic taxonomy

The workflow for polyphasic taxonomy of newly isolated strains, included genotype and phenotype analysis. A flow diagram of the taxonomical characterizations is shown in Figure 2-4.

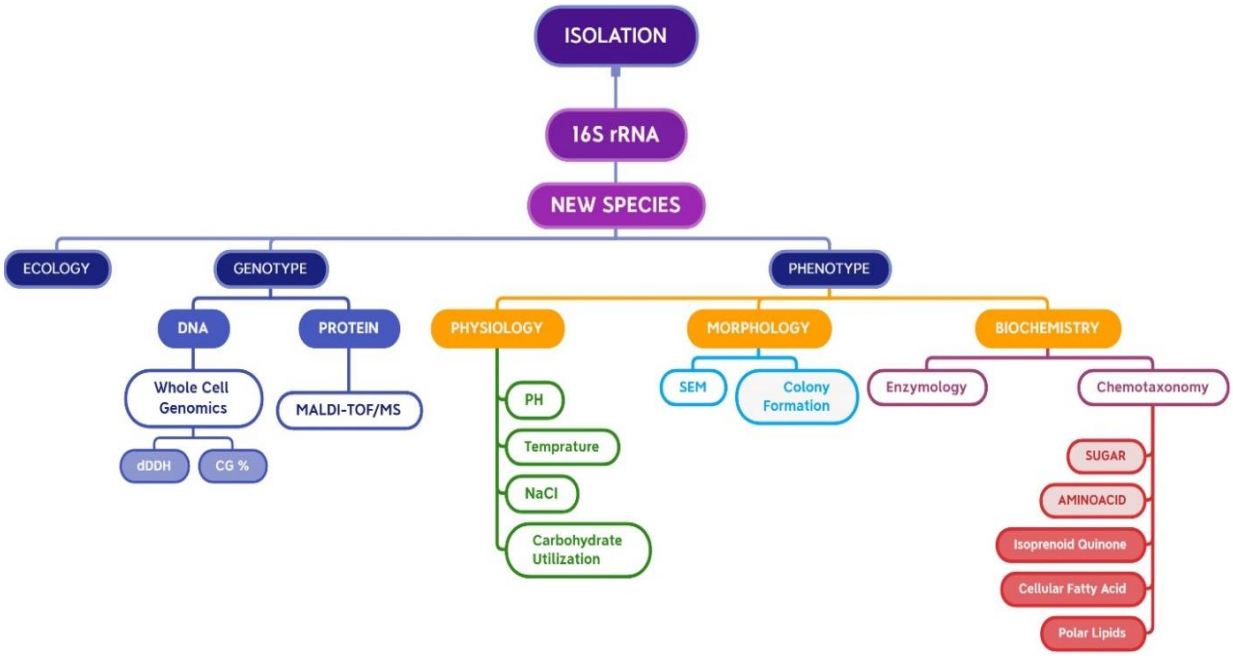


Figure 2-4 Flow diagram for taxonomical characterization of newly isolated strains

- **Genotype**

Genomic DNA of strains was extracted following the protocol described by Jin *et al*. (97). Libraries for Whole-Genome Sequencing on the Illumina platform were prepared from extracted genomic DNA applying the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, USA). Samples were sequenced using the Illumina NextSeq™ 500 by GMAK and DSMZ. The digital DNA-DNA Hybridization (dDDH) values and G+C content between the genome sequences of strains and their type strains were calculated using the Genome-to-Genome Distance Calculator GGDC 2.1 (http://ggdc.dsmz.de) (42).

MALDI-TOF/MS analyses were performed in German collection of microorganisms and cell cultures (DSMZ) with the type strains. The sample preparation was done by applying ethanol/formic acid extraction as described in Protocol 3 of Schumann & Maier (2014) (43).

- **Phenotype**

Phenotype included physiological, morphological and biochemical analysis.

- **Physiology**

The pH range of 5.0 to 10.0 (intervals of 1 pH unit) were determined on GYM agar by incubation at 30 ˚C for 1 week. Growth at temperatures of 20, 30, 35 and 40 ˚C were observed on GYM agar after incubation for a week. The resistance towards sodium chloride is taken out by analysing the growth on basal medium in a 6-well flat bottom plate with 0, 2.5, 5, 7.5 and 10 % concentration of sodium chloride respectively(98). 10 % stock solutions of 10 different carbon sources were added to the basal medium to test the ability of strains to utilise them (99). The final concentration of 1% was applied in a 12-well flat bottom plate. Carbohydrates that were used as carbon sources included: glucose, arabinose, sucrose, xylose, inositol, mannitol, fructose, rhamnose, raffinose, and cellulose (Figure 2-5).

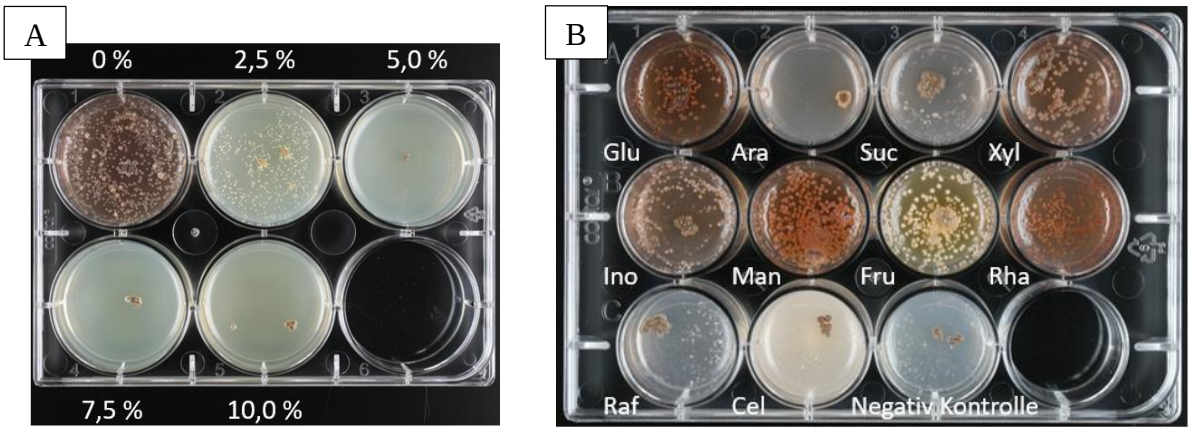


Figure 2-5 Actinomycetes strain on A) salt plates B) carbon source plates

- **Morphology**

For scanning electron microscopy analysis, a section of the ISP2 and ISP3 plate (1 cm x 1 cm) containing bacteria with aerial mycelium were fixed in 5 % aqueous glutaraldehyde. The plates were incubated for at least 14 days at 30 ˚C (100). The images were recorded with SmartSEM software version 6.06 by Prof. Dr. Manfred Rohde, HZI Braunschweig.

Colony formation, description and melanin production were tested by culturing the bacteria on ISP2, ISP3, ISP4, ISP5, ISP6, ISP7 media (International Streptomyces Project 2), SSM+T (synthetically Suter medium with tyrosine) and SSM-T (synthetically Suter medium without tyrosine)(Figure 2-6). 500 µl of the culture was plated out and the

plates were incubated for 10 to 14 days at 28 ˚C or otherwise to the temperature required for the given strain. Four parameters, growth, reverse color, aerial mycelium and soluble pigment were determined by comparison with the RAL-code (RAL colour code 1005; Deutsches Institut für Gütesicherung und Kennzeichnung e. V. – Reichs-Ausschuss für Lieferbedingungen)(Figure 2-7).

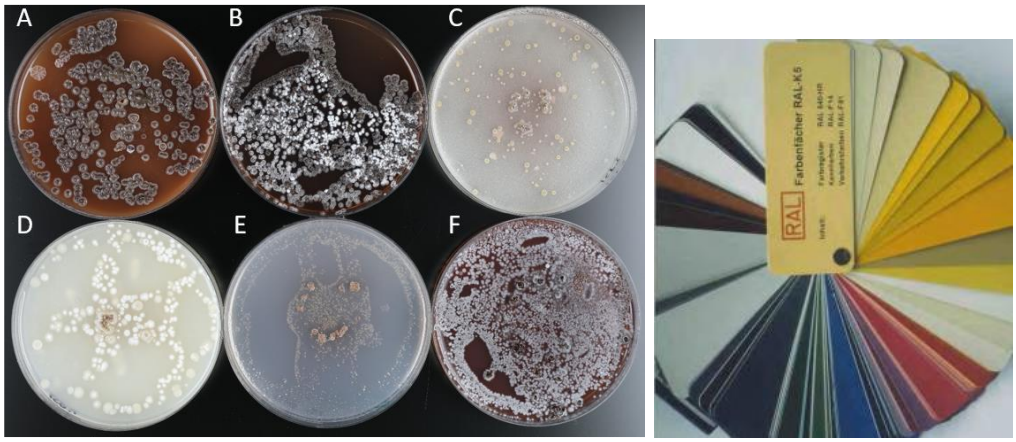


Figure 2-6 Colony description based on RAL(A:GYM, B:ISP2, C:ISP3, D:ISP4, E:ISP5, F:ISP7)

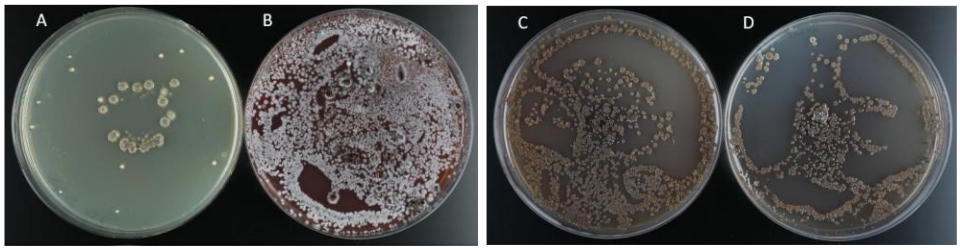


Figure 2-7 Formation of melanin pigment (A:ISP6, B:ISP7, C:SSM+, D:SSM-)

- **Biochemistry**
  - Enzymology: The enzymatic activities were determined using API®ZYM and API® Coryne test strips (BioMérieux) (101,102)
  - Chemotaxonomy: For performing chemotaxonomic analyses like polar lipids, fatty acid, cell-wall quinones, sugars and amino acids, freeze dried cells are needed. The wet biomass was obtained from 7 days old GYM liquid culture by centrifuging at 9000 rpm for 5 min. Cells were harvested and washed three times with sterile distilled water. Then the wet biomasses were lyophilized.

Table 2-5 Semiquantitation of enzymatic activities

API®ZYM	API® Coryne
Alkaline phosphatase	Nitrate reduction
Butyrate esterase (C4)	Pyrazinamidase
Caprylate esterase lipase (C8)	Pyrrolidony arylamidase
Myristate lipase (C14)	Alkaline phosphatase
Leucine arylamidase	β-glucuronidase
Valine arylamidase	β-galactosidase
Cystine arylamidase	α-Glucosidase
Trypsin	N-acetyl-β-glucosamidase
α-Chymotrypsin	Esculin (β-glucosidase)
Acid phosphatase	Urease
Naphtol-AS-BI-phosphohydrolase	Gelatine (hydrolysis)
α-Galactosidase	Glucose fermantation
β-Galactosidase	Ribose fermentation
β-Glucoronidase	Xylose fermentation
α-Glucosidase	Mannitol fermentation
β-Glucosidase	Maltose fermentation
N-acetyl-β-glucosaminidase	Lactose fermentation
α-Mannosidase	Sucrose fermentation
α-Fucosidase	Glycogen fermentation

Aminoacid: For amino acids extraction, a 5 mg of freeze-dried sample was hydrolyzed in 1 ml of 6N HCl and incubated in a water bath at 100 ºC for 18 h. The hydrolysate was dried to remove the HCl and the residue dissolved in 0.3 ml of distilled water. Amino acids were identified by Thin Layer Chromatography (TLC) using the solvent mixture methanol-water-10N HCl-pyridine (80:17.5:2,5:10 v/v). The paper was sprayed with ninhydrin solution and heated at 100 ºC (103).

Sugar: For sugars extraction, a 10 mg (dry weight) sample was hydrolyzed in 1 ml of 2N H_2SO_4 and incubated in water bath at 100 ºC for 2 h. The hydrolysate was neutralized to pH 5.0 to 5.5 with $Ba(OH)_2$. After centrifuging at 1800 rpm for 5 min, the liquid phase was evaporated under reduced pressure and the residue dissolved in 0.4 ml H_2O. The sugars were separated by TLC, using the mixture of solvents consisting of ethyl acetate-pyridine-water (3.6:1:1.15 v/v). The plate was sprayed with aniline phthalate before heating it at 100 ºC for 5 min (103).

Menaquinone: For menaquinones extraction, a 100 mg of freeze-dried sample was mixed with 4 ml methanol-0.3% aqueous NaCl (90:10) and 4 ml petroleum ether. After 30 min of mixing on a tube rotator, the upper phase was transferred and evaporated at 30 ºC at reduced pressure. The dry extract was dissolved with 100 µl acetonitrile-isopropanol (65:35 v/v) (104). Respiratory quinones were analysed by an HPLC equipped for diode-array detection and mass spectrometry (HPLC-DAD-MS)(105).

Polar lipids: For polar lipids extraction, a 200 mg of freeze-dried sample was mixed with 8 ml of Chloroform-MeOH (2:1) and rotated for 16 h. The suspension was filtered with filter paper and evaporated at reduced pressure at 30 ºC. The extract was dissolved with 200 µl Chloroform-MeOH (2:1). Two-dimensional thin-layer chromatography was applied on HPTLC Kieselgel 60F_{254} (Merck) plates with two mixtures of solvents. The mixture of solvents used for the first dimension consisted of chloroform/methanol/water (65:25:4 v/v) and the second mixture included chloroform/methanol/ acetic acid/water (80:12:15:4 v/v) (105). Detection of polar lipids was surveyed using different reagents (Table 2-6).

Table 2-6 List of reagents for phospholipid analysis

Reagent	Composition	Heat	Identification
Molybdophosphoric acid	20 % (v/v) in Ethanol	140 °C, 15 min	All lipids
Ninhydrin	Merck 6758	100 °C, 4 min	Free NH_4
Molybdenum blue	Sigma M-3389		Phosphate-containing lipids PO_4
Anisaldehyde	0.5 ml Anisaldehyde, 0.5 ml H_2SO_4, 9 ml 96% Ethanol	100 °C, 10 min	Sugar-containing lipids
α-Naphtol-H_2SO_4	10.5 mL 15 % (w/v) α-Naphtol in Ethanol, 6.2 ml H_2SO_4, 40.5 ml Ethanol, 4 ml H_2O	120 °C	

Fatty Acid: For fatty acid extraction, a 10 mg of freeze-dried sample were mixed with 1ml saponification reagent (45 g NaOH, 150 ml MeOH, 150 ml H_2O) and heated in a water bath at 100 ºC for 5 min. After vortexing, 2 ml of methylation reagent (325 ml

6N HCl, 275 ml MeOH) was added to the mixture. The capped tube was heated at 80 ºC for 10 min. 1.25 ml of extraction reagent (200 ml hexane and 200 ml Methyl tert-butyl ether (MTBE)) was added to mixture and rotated for 10 min. After discarding the lower phase, 3 ml of washing reagent (10.8 g NaOH, 900 ml H_2O) was added to the organic phase. After tumbling for 5 min, the upper phase was transferred to a GC vial. The GC was performed and analysed using the Sherlock Microbial Identification (MIDI) system and the ACTIN version 6 database (106).

2.7.1.2 Fermentation and compound isolation of strain 4NS15

- Optimization of medium, volume and harvesting day:

In order to determine the optimal medium, volume and harvesting day, different conditions applied for obtaining the raw extract of strain 4NS15. Four media: 5254, 5294, 5333 and soy meal were chosen and various volumes: 100, 250, 500, 750 ml and 1 liter were tested. Growth and production of the specific metabolites were screened on different days. From day 7 to 15, the cells were harvested and their crude extract was evaluated using HR-ESI-MS.

- Fermentation:

To extract the target compounds from the strain 4NS15, 100 ml of 7 days old sub-cultured GYM broth was inoculated in each 2 L Erlenmeyer flask containing 1 L of 5294 medium (10 flasks). The cultures were incubated in a rotary shaker at 160 rpm at 30 ºC for 15 days. A total volume of 10 L was centrifuged and the pellet and supernatant separated. 5% XAD 16 was added to the supernatant and left shaking for 24 hours. The XAD resin from supernatant was collected by sieving, washed by flushing with distilled water, and then extracted three times with methanol. Pellet was extracted with acetone, then sonicated, filtered and evaporated in a rotary evaporator.

- Compound isolation:

20 µl aliquots of the extracts were injected onto an analytical size luna5µm C18 (250×4.6 mm) fitted with a guard-column. The compounds were separated by multi gradient elution using water with 0.1% formic acid as solvent A and acetonitrile with 0.1% formic acid as solvent B at a flow rate of 1 ml/minute. The isolation process was done in Helmholtz Institute for Pharmaceutical Research Saarland in Saarbrücken under supervision of Dr. Emilia Queis.

- Cytotoxic assay:

A cytotoxicity test was conducted for 1 mg/ml of pure compound isolated from 4NS15 (Fraction 3) in MeOH by applying the MTT method (107). Eight tested cell lines were: L-929 (murine fibroblast), KB-3-1 (human cervix carcinoma), A-549 (human lung carcinoma), PC-3 (human prostate carcinoma), MCF-7 (human breast adenocarcinoma), A-431 (human epidermoid carcinoma), SK-OV-3 (human Caucasian ovary adenocarcinoma) and HUVEC (human umbilical vein endothelial cell). Methanol was used as a negative control. The cytotoxic assay was performed by Mrs. Wera Collisi, HZI-Braunschweig.

- Structure elucidation:

To elucidate the structure of the isolated compounds from strain 4NS15, the measurements of HRMS, MS^2 fragmentation and NMR were performed in HIPS (Helmholtz Institute for Pharmaceutical Research Saarland in Saarbrücken) by Dr. Emilia Queis. The data is still under investigation by Dr. Lena Keller.

2.7.1.3 Dead end for two strains NS40 and NS37

Multilocus Sequence Analysis (MLSA) was done for two strains NS40 and NS37 with three housekeeping genes *gyrB*, *atpD* and *trpB* according to Guo *et al*. (108) (Table 2-7).

Table 2-7 The list of primers for multi locus sequencing

Gene	Primer	Sequence (5' -> 3')
atpD	*atpD*PF (amplification)	GTCGGCGACTTCACCAAGGGCAAGGTGTTCAACACC
	*atpD*F (sequencing)	ACCAAGGGCAAGGTGTTCAA
	*atpD*PR (amplification)	GTGAACTGCTTGGCGACGTGGGTGTTCTGGGACAGGAA
	*atpD*R (sequencing)	GCCGGGTAGATGCCCTTCTC
gyrB	*gyrB*PFA (amplification)	TCGAGGGTCTGGACGCGGTCCGCAAGCGACCCGGTATG TA
	*gyrB*FA (sequencing)	GCAAGCGACCCGGTATGTAC
	*gyrB*PAR (amplification)	GAAGGTCTTCACCTCGGTGTTGCCCAGCTTCGTCTT
	*gyrB*RA (sequencing)	GAGGTTGTCGTCCTTCTCGC
trpB	*trpB*PF (amplification)	GCGCGAGGACCTGAACCACACCGGCTCACACAAGATCA ACA
	*trpB*F (sequencing)	GGCTCACACAAGATCAACAA
	*trpB*PR (amplification)	TCGATGGCCGGGATGATGCCCTCGGTGCGCGACAGCAG GC
	*trpB*R (sequencing)	TCGATGGCCGGGATGATGCC

The PCR amplification were performed using touch down TD60 strategy (109). The sequences of the three housekeeping genes were aligned using MUSCLE (multiple sequence comparison by log-expectation) (110) in MEGA X software (111). Then phylogenetic trees calculations were performed using GGDC.

- NS40

- Polyphasic taxonomy:

Polyphasic assays were performed on NS40 and closely related type strains that were suggested by the EzTaxon database. *Streptomyces badius* DSM40139^T, *Streptomyces sindenensis* DSM40255^T, *Streptomyces parvus* DSM40348^T were obtained from the German collection of microorganisms and cell cultures (DSMZ) for comparison under the same conditions. DDH has been done by Dr. Kämpfer in Gießen.

- Antibacterial assay, screening for secondary metabolites and fermentation:

The antibacterial activity was determined with serial dilution assay. HPLC fractionation and LC/MS results were analysed and compared in DNP. To extract the target compounds from NS40, the bacterial strain cultured in 50 flasks containing 100 ml of 5294 medium in 250 ml Erlenmeyer shaken for 7 days at 28 °C (a total of 5 L). After centrifugation, 5% XAD was added to the supernatant and left shaking for 24 hours, after which XAD was separated and extracted three times with methanol, whereas the pellet was extracted with ethyl acetate.

- NS37

- Polyphasic taxonomy:

Some parts of physiology, morphology and biochemistry tests were performed with closely related type strains, including: *Nocardiopsis umidischolae* DSM44362^T and *Nocardiopsis tropica* DSM44381^T. DDH analysis was done for the strains NS37 and DSM44362^T in Gießen by Dr.Kämpfer. MALDI-TOF was done for the strain in DSMZ.

- Antiviral assay

The antiviral assay was conducted with crude extract of NS37 according to Mulwa *et al.* (112) by Prof. Dr. Eike Steinmann in TWINCORE-Hannover.

2.7.2 Myxobacteria

- **4NSX3**

Among different isolated myxobacteria, strain 4NSX3 was chosen to screen for its secondary metabolites. The pure strain was cultured in CYH broth medium. After 10 days, 10% of CYH transferred to four media: myxoverisin, P, POL and H that contained 2% XAD. After 10 days of incubation, the XAD in the media harvested with aceton and the crude extracts were analysed with HPLC and HR-ESI-MS. Later the compounds were compared against the Myxobase.

2.7.3 Actinobacteria from snail

2.7.3.1 7NS3

From all 28 isolated Actinobacteria from snails, 7NS3 was chosen for characterization and metabolite profiling. The strain 7NS3 was isolated from the freshwater snail *Physa acuta*, which was collected from a pond (52°6′42.32″ N, 10°40′46.20″ E) in Remlingen-Semmenstedt, Germany in June 2019.

- Polyphasic taxonomy

The whole-genome sequencing was performed in DSMZ and all polyphasic taxonomy approaches completed.

- BGC identification and metabolite profiling

The potential biosynthetic gene clusters for producing novel compounds from this isolate were detected by bioinformatics assay antiSMASH and analysed by Prof. Dr. Mast in DSMZ (113).

3 Results

3.1 Soil samples

3.1.1 Actinobacteria

Eighty Actinobacteria were isolated from soil samples of nine cities in Iran and the city of Braunschweig in Germany (Table S2). The soil samples were collected from different plant leaves (Mord, Khesht), the root-soil of special trees and plants (Almond, Oak, Astragalus, Safran and Lichen), the sand of beaches and the deserts. Most of the isolates were from the city Yasooj, but the most diverse isolation source in terms of rare Actinobacteria was the soil sample from the desert in Isfahan because of applying pretreatment to the soil. Based on partial 16S rRNA gene sequencing of 80 isolates, almost 70% of the isolates belonged to the genus *Streptomyces*, and the rest were characterized as non-*Streptomyces* (Figure 3-1).

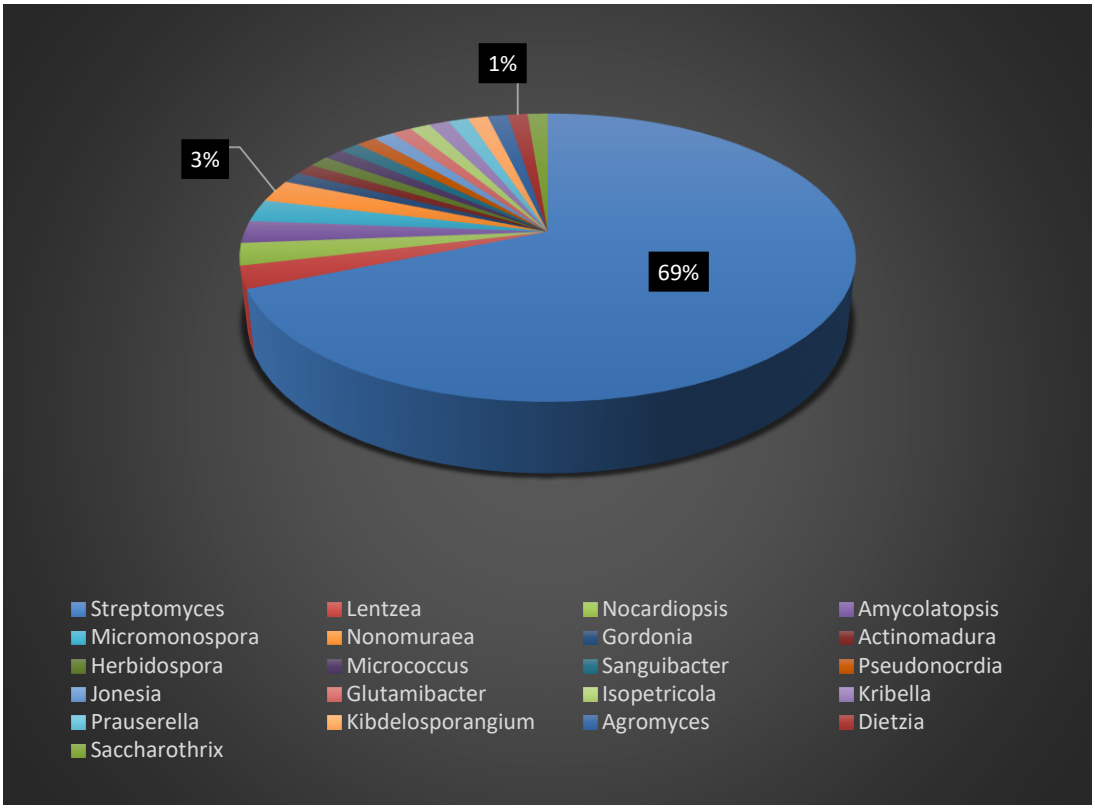


Figure 3-1 Genus-level diversity of Actinobacteria isolated from soil samples

Among all isolates, fifteen strains that belonged to more interesting families according to the taxonomy were chosen for full 16S rRNA gene sequencing (Table 3-1) and among them, five were targeted for characterization by the polyphasic approach and

whole-genome sequencing to characterize as novel species. The list of these top five isolates with their accession numbers is shown in Table 3-2.

Table 3-1 Top 15 and 5 targeted Actinobacteria (in red)

Targeted samples	16S rRNA Similarity (full sequence %)	
NS40	*Streptomyces badius*	98.55
NS36	*Lentzea violacea*	99.10
NS66	*Amycolatopsis albidoflavus*	99.79
NS37	*Nocardiopsis umidischolae*	99.58
NS38	*Nonomuraea harbinensis*	99.65
NS25	*Actinomadura apis*	99.23
Q1	*Amycolatopsis rifamycinia*	99.03
Q2	*Pseudonocardia cypriaca*	99.65
NS34	*Kribbella pittospori*	99.85
NS59	*Streptomyces specialis*	98
NS44	*Herbidospora sakaeratensis*	97.64
44ZA	*Sanguibacter keddieii*	98.34
BA	*Agromyces arachidis*	99.08
Sef	*Prauserella alba*	100
4NS15	*Kibdelosporangium aridum*	98.96

Table 3-2 Targeted isolates (Top5) with accession numbers

Code	Accession number
Q1	DSM110486-CIP111796
Q2	DSM110487-CIP111797
44ZA	DSM110740-CIP111798
BA	DSM111422-NCCB100802
4NS15	DSM110728-CIP111705-NCCB100701

Polyphasic taxonomy, identification or classification is a set of different methods applied to get reliable taxonomy of bacteria. In the following part, the results of the polyphasic taxonomy (genotype and phenotype), some information about ecology, whole-genome sequencing and the ability to produce secondary metabolites of the mentioned five top strains are presented.

3.1.1.1 Strains Q1 and Q2

- Ecology

Isolates Q1 and Q2 were isolated from silver beach sand in Hengam island located south of Qeshm island in the Persian Gulf. The pH of the sand without ion exchanger is 8.49 and with (CaCl2) is 8.28.

- Genotype

Analysis of the complete 16S rRNA gene sequencing of the two strains Q1 and Q2 in the EzTaxon database suggested that they are closely related to the type strains shown in Table 3-3. Strain Q1 showed 16S rRNA gene sequence similarity of 99.03% to the type strain of *Amycolatopsis rifamycinica* DSM 46095 and strain Q2 with 99.65% showed the highest similarity to *Pseudonocardia cypriaca* DSM 45511.

Table 3-3 Q1 and Q2 full 16S rRNA gene sequecning in the EzTaxon database

Strain	Type strains	Similarity based on 16S rRNA gene	DSM code
Q1 DSM 110486 *Amycolatopsis*	*A. rifamycinica*	99.03%	DSM 46095
	A. rhabdoformis	98.96%	SB026-no DSM
	A. kentuckyensis	98.89%	DSM 44652
	A. lexingtonensis	98.82%	DSM 44653
	A. pretoriensis	98.75%	DSM 44654
	A. balhimycina	98.68%	DSM 44591
Q2 DSM 110487 *Pseudonocardia*	*P. cypriaca*	99.65%	DSM 45511
	P. hierapolitana	99.45%	DSM 45671
	P. zijingensis	99.29%	DSM 44774
	P. adelaidensis	99.15%	DSM 45352

Pairwise 16S rRNA gene sequence similarities, as well as the maximum-likelihood (ML) and maximum-parsimony (MP) trees, were carried out using the DSMZ phylogenomic pipeline of the Genome to Genome Distance Calculator server (GGDC). The results showed that strain Q1 has the highest similarity to *Amycolatopsis alba* DSM 44262 (Figure 3-2) and *A. rifamycinica* DSM 46095 is in a different clade far from Q1. On the other hand, strain Q2 showed the highest similarity to *P. cypriaca* DSM 45511, which was in line with the EzTaxon database result (Figure 3-3).

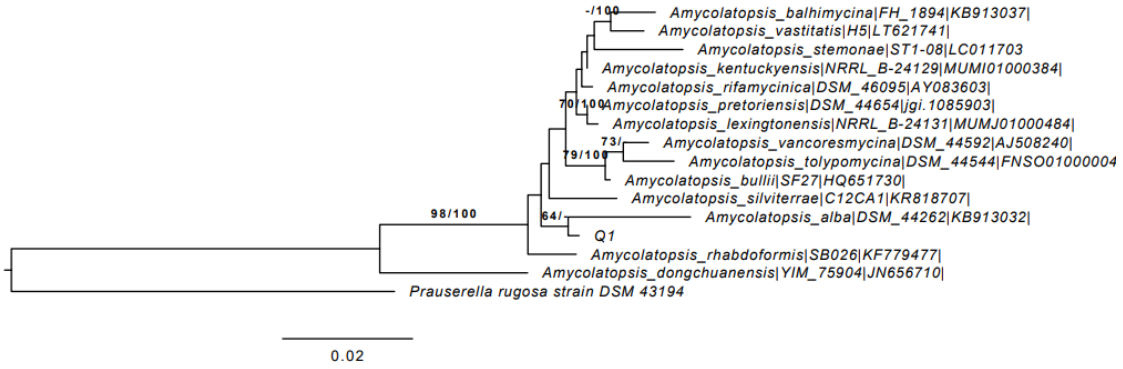


Figure 3-2 Maximum-likelihood phylogenetic tree based on full-length 16S rRNA gene sequence of Q1 and most related species. Numbers above the branches are bootstrap support values greater than 60% for maximum-likelihood (left) and maximum-parsimony (right) method. Bar, 0.02.

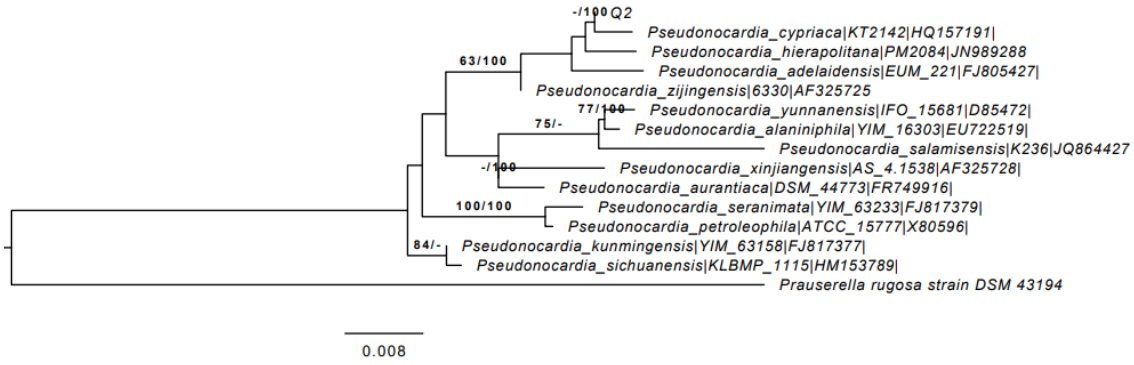


Figure 3-3 Maximum-likelihood phylogenetic tree based on full-length 16S rRNA gene sequence of Q2 and most related species. Numbers above the branches are bootstrap support values greater than 60% for maximum-likelihood (left) and maximum-parsimony (right) method. Bar, 0.008.

Whole-genome sequencing for both strains were achieved by PacBio-sequencing performed in the DSMZ. The draft genome sequence of Q1 has the size of 11.03 Mb with an average G+C content of 70,28% and Q2 has 10.32 Mb with 71,77% G+C content. Digital DNA-DNA hybridization (dDDH) values (formula d_4) showed Q1 is related to the type strain *Amycolatopsis saalfeldensis* DSM 44993^T with 29.0% and Q2 is similar to the type strain *Pseudonocardia hierapolitana* DSM 45671^T with the value of 46.1%.

MALDI-TOF dendrogram results showed Q1 and *Amycolatopsis rhabdoformis* SB026 grouped in one cluster with a distance level of 600. Due to lack of data in the database, there was no data for the most similar type strains of *A. rifamycinica* DSM 46095 and *A. saalfeldensis* DSM 44993^T. For strain Q2, the d4endrogram showed the highest

similarity between Q2 and *P. cypriaca* DSM 45511 with a distance level of 350 (Figure S1).

➢ Phenotype

- Physiology

Optimal growth for both strains were observed on GYM medium plates at a temperature of 30 °C. Both strains have good growth at pH 8, 9, and 10. Q1 was able to grow in presence of 5 % NaCl, but Q2 was not able to grow with NaCl. Q1 utilised all tested carbohydrates as carbon sources except cellulose (Figure S2). Q2 grew with the tested carbon sources, but no growth was seen in plates with raffinose and cellulose (Figure S3).

- Morphology

No scanning electron microscopy were captured from both strains because they formed no aerial mycelium. The special morphology on GYM medium is shown in Figure 3-4. Strain Q1 grew well on all ISP plates but no pigment production was seen in ISP 2, ISP 5, ISP 6, ISP 7, SSM+T and SSM-T media (Figure S4). No growth was observed for Q2 in ISP 4 and 5. No melanin was produced in the melanin production media (Figure S5).

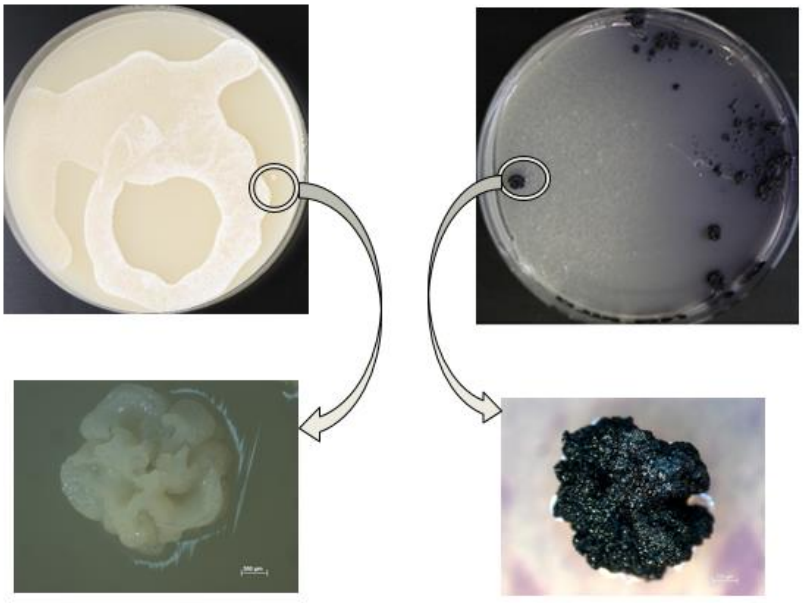


Figure 3-4 Q1 and Q2 morphology on GYM

- Biochemistry

Q1 and Q2 possessed good enzymatic activities for β-glucoronidase, which showed a remarkable difference between the two strains and their type strains (Figure S6). The differences in API ZYM and API Coryne are shown in Table 3-4.

Table 3-4 Differences in API ZYM and API Coryne; of Q1 and relative type strains

Enzyme	Q1	DSM 46095	SB 026	DSM 44652	DSM 44653	DSM 44654	DSM 44591
α-chymotrypsin	-	+	-	+	+	+	-
β-glucoronidase	+	-	+	-	-	-	-
α-fucosidase	-	-	-	+	+	+	+
Nitrate reduction	-	-	-	-	+	-	+
Esculin	-	+	+	+	+	+	-
Gelatine (hydrolysis)	+	+	+	+	+	+	-

For Q2 esculin and gelatin liquefaction was positive, which was different from other type strains (Figure S7). Differences in API ZYM and API Coryne are shown in Table 3-5.

Table 3-5 Differences in API ZYM and API Coryne of Q2 and relative type strains

Enzyme	Q2	DSM 45511	DSM 45671	DSM 44774	DSM 45352
Alkaline phosphatase	++	+	+	-	++
Butyrate esterase (C4)	-	+	-	+	-
α-galactosidase	+	-	+	-	+
β-glucosidase	+	-	-	-	-
β-glucoronidase	+	-	-	-	-
Nitrate reduction	+	-	+	-	+
Pyrazinamidase	+	-	+	-	+
Esculin	+	-	-	-	-
Gelatine (hydrolysis)	+	-	-	-	-

- Chemotaxonomy

Both strains contained *meso*-diaminopimelic acid, arabinose and galactose in whole-organism hydrolysates and strain Q2 contained glucose and ribose as well. The predominant isoprenologue in Q1 was tetrahydrogenated menaquinone with nine

isoprene units MK-9(H_4) and for Q2 was tetrahydrogenated menaquinone with eight isoprene units MK-8(H_4) (Figure S8). The polar lipid composition in Q1 was identified as diphosphatidyl-glycerol (DPG), phosphatidyl-N-methyl-ethanolamin (PME), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). For Q2 also DPG, PE, PG and phosphatidylcholin (PC) were identified (Figure S9). The major cellular fatty acids pattern of Q1 and Q2 consisted mainly of iso-C16:0 (40% and 42% respectively) and in this regard Q1 was similar to two type strains of *A. rifamycinica* DSM 46095 and *A. balhimycina* DSM 44591 (Table 3-7) and strain Q2 had higher similarity to *P. cypriaca* DSM 45511 and *P. hierapolitana* DSM 45671. The major fatty acids of strain Q1 and Q2 and their type strains are shown in Table 3-6 and

Table 3-7.

Table 3-6 Cellular fatty acid composition of strain Q1

Fatty acid	Q1	DSM 46095	SB 026	DSM 44652	DSM 44654	DSM 44653	DSM 44591
saturated straight chain							
C14:0	0.91						
C15:0	2.52	2.17	1.63				
C16:0	8.27	7.91	7.67	5.64	6.83	4.82	3.04
C17:0	2.68	6.14	4.29				
C18:0	1.85		2.6				
C21:0	2.68						
saturated branched chain							
iso- C14:0	4.78	1.63	2.23	2.29	3.08	2.17	1.85
iso- C15:0	11	7.67	27.84	26.82	31.42	38.03	9.39
iso- C16:0	40.5	40.82	27.11	14.58	16.3	7.5	39.33
iso- C17:0	1.53	2.27	5.98	6.28	6.39	6.45	3.89
anteiso- C15:0		1.53	2.6	31.13	23.27	32.19	
anteiso- C17:0	0.99	6.18	5.25	13.25	9.91	8.85	3.61
cy C17:0	3.6						
C17:0 10- methyl		1.59					1.9
iso- C18:0	0.83						
unsaturated chain							
C16:1ω7	6.6	5.56	9.24				7.16
C17:1ω8	0.83	2.31					2.23
C17:1ω6	9.63	10.42	3.56				15.94
iso- C16:1ω6							5.31
iso- C17:1ω7		2.17			2.79		2.85
iso- c/t C17:1ω7							3.51

c/t C18:1ω7	0.78						
C18:1ω5		1.63					

Table 3-7 Cellular fatty acid composition of strain Q2

Fatty acid	Q2	DSM45511	DSM45671	DSM44774	DSM45352
saturated straight chain					
C13:0			0.3		
C14:0			0.79		
C15:0	1.84	1.21	6.76	2.01	
C16:0	5.64	6.72	7.34	3.49	7.22
C17:0	2.27	2.61	6.39	2.39	
C18:0					1.12
saturated branched chain					
C14:0 3- hydroxy	1.04	0.69	0.49		
iso- C14:0	1.77	0.69	2.6	2.46	1.67
iso- C15:0	8.29	5.81	8.01	5.87	38.35
iso- C16:0	42.48	33.01	36.6	51.8	6.83
iso- C17:0	3.36	4.95	2.54	6.44	7.55
anteiso- C15:0	1.86	1.4	2.92	1.33	23.8
anteiso- C17:0	9.6	14.2	9.56	5.84	8.01
C17:0 10- methyl	4.48	4.19	2.74	6.4	
iso- C18:0	0.6		0.29		
unsaturated chain					
C15:1ω6	0.51		1		
C16:1ω5				2.73	
C16:1ω7	1.61	5.49	1.79		
C17:1ω8	2.77	5	5.27	2.05	
C17:1ω6	1.26	2.17	1.05		
iso- C16:1ω6	1.92	2.24	1.29	1.25	
iso- C16:1ω8				2.01	
iso- C17:1ω7	1.28	8.37	1.86	3.94	
iso- c/t C17:1ω7	6.91	1.26	0.39		
C18:1ω9					2.63
C18:2ω6,9	0.51				2.82

➢ Secondary metabolites

Both strains had weak activity against Gram-positive bacteria. The crude extracts derived from Q2 in 4 metabolite media (5254, 5294, soy meal, 5333) were analysed using high-resolution electrospray ionization-mass spectrometry (HR-ESI-MS). The main peak with the retention time (t_R)=11 min, which was correlated to activity in the HPLC chromatogram showed ion at a mass-to-charge ratio (*m/z*) of 394.06 corresponding to $(M+H)^+$. There were 10 hits in the Dictionary of Natural Products, mostly belonging to the class of terpenoids like capparisesterpenolide A. No hits were found from the phylum Actinobacteria. The raw extract of Q1 showed a mass with specific UV absorption of 212, 255, 275, 335 nm with ion *m/z* 772.66 corresponding to $(M+H)^+$. No hits were found in the DNP.

3.1.1.2 44ZA and BA

➢ Ecology

among different soil samples, the soil of Semnan desert was chosen to isolate just non-*Streptomyces* group of Actinobacteria (rare Actinobacteria). The soil was treated with different methods of pretreatment like adding chloramine T or heating the soil up to 60 ˚C for 30 minutes and using different and specific media for isolation. The list of rare Actinobacteria isolated after pretreatment are shown in Table 3-8. Two strains 44ZA and BA, were chosen for polyphasic taxonomy.

Table 3-8 List of rare Actinobacteria isolated from Isfahan soil after treatment

Isfahan	NS38	Desert soil	*Nonomuraea harbinensis*	99.62
	NS44	Desert soil	*Herbidospora sakaeratensis*	97.64
	NS45	Desert soil	*Micromonospora echinofusca*	99.63
	44ZA	Desert soil	*Sanguibacter keddieii*	98.34
	BA	Desert soil	*Agromyces arachidis*	99.08
	441B	Desert soil	*Goordonia alkanivomans*	100
	Sef	Desert soil	*Prauserella alba*	100
	SO	Desert soil	*Dietzia maris*	99.7
	44MX	Desert soil	*Jonesia quighaiensis*	100
	44Z	Desert soil	*Micrococcus aloverae*	100

The different morphology of isolated rare Actinobacteria in two media was shown in Figure 3-5. For instance, four isolates NS38 (*Nonomuraea)*, 441B *(Goordonia)*, 44MX

(*Jonesia)* and 44ZA *(Micrococcus)* showed quiet similar colonies with colors ranged from white to yellow in 5336 medium, which was different from GYM medium. On GYM medium, the colonies of NS38 (*Nonomuraea),* 441B *(Goordonia)* were orange and soft, formed bright orange color. For 44MX (*Jonesia)* and 44ZA *(Micrococcus)* on GYM medium, the colonies were bright yellow and soft.

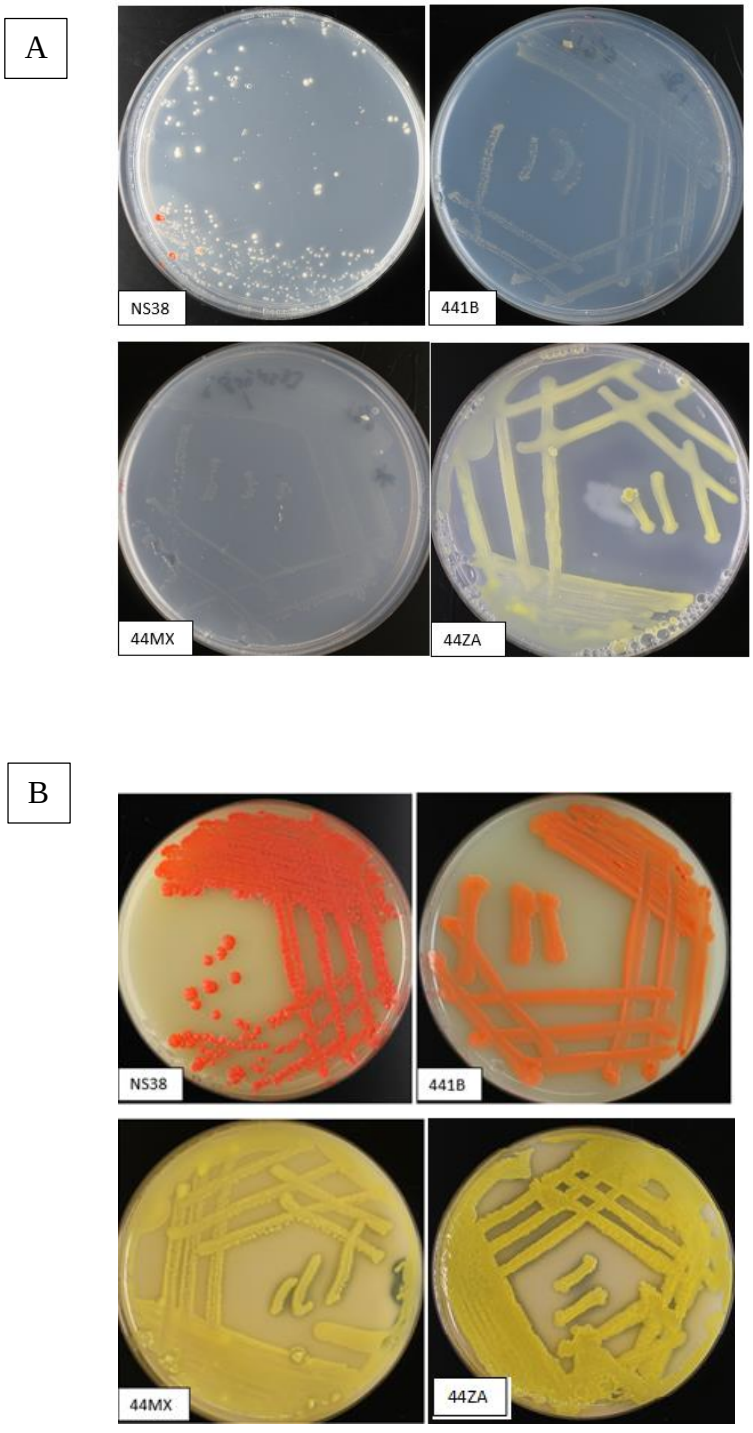


Figure 3-5 Different morphologies of four isolates NS38 (*Nonomuraea)*, 441B *(Goordonia)*, 44MX (*Jonesia)* and 44ZA *(Micrococcus)* in 5336 medium (A) and GYM medium (B)

- Genotype

based on the complete 16S rRNA gene sequencing, NS44ZA was found to be closely related to the type strain *Sanguibacter keddieii* with similarity of 98.34 %. The strain BA was closely related to *Agromyces arachidis* with 99.08 % and *Agromyces indicus* with similarity of 98.80 %. The type strain *A. arachidis* is not validly published, therefore, the analyses were done with the type strain *A. indicus*, which was close to *A. arachidis* (Table 3-9).

Table 3-9 Complete 16S rRNA gene sequencing results of NS44ZA and BA

Strain	Type strains	Similarity based on complete 16S rRNA gene	DSM code
NS44ZA *Sanguibacter* DSM 110740	*S. keddieii*	98.34%	DSM 10542
BA *Agromyces* DSM 111422	*A. indicus*	98.80%	DSM 26681

Whole-genome sequencing was performed using Illumina MiSeq (PE300) platform for both strains by Genomanalytik (GMAK) at HZI. The draft genome of NS44ZA consisted of 37 contigs with a total consensus of 3,475,186 bp and an average G+C content of 72.08 %. The digital DNA-DNA hybridization (dDDH) value between NS44ZA and *S. keddieii* was 22.2 %, which was below the threshold 70 %. Average nucleotide identity value was <90 % (77 %), which was below the threshold 94-96 %. The maximum-likelihood phylogenetic tree of NS44ZA revealed a higher similarity of the strain to two type strains: *S. keddieii* and *S. inulinus* (Figure 3-6).

The whole-genome sequence of BA consisted of 88 contigs with a total size of 3,870,556 bp and an average G+C content of 71.53 %. The phylogenetic tree was performed using the TYGS server. The results revealed that no specific type strain was closely related to the strain BA (Figure 3-7).

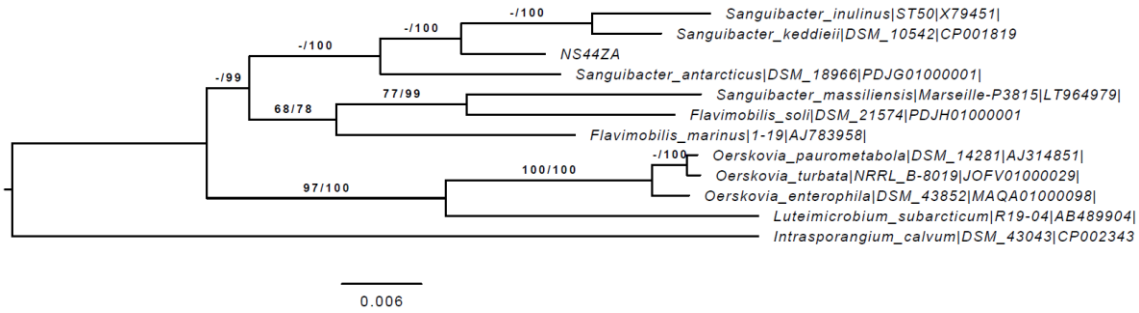


Figure 3-6 Maximum-likelihood phylogenetic tree based on full-length 16S rRNA gene sequence of NS44ZA. Numbers above the branches are bootstrap support values greater than 60% for maximum-likelihood (left) and maximum-parsimony (right) method. Bar, 0.006.

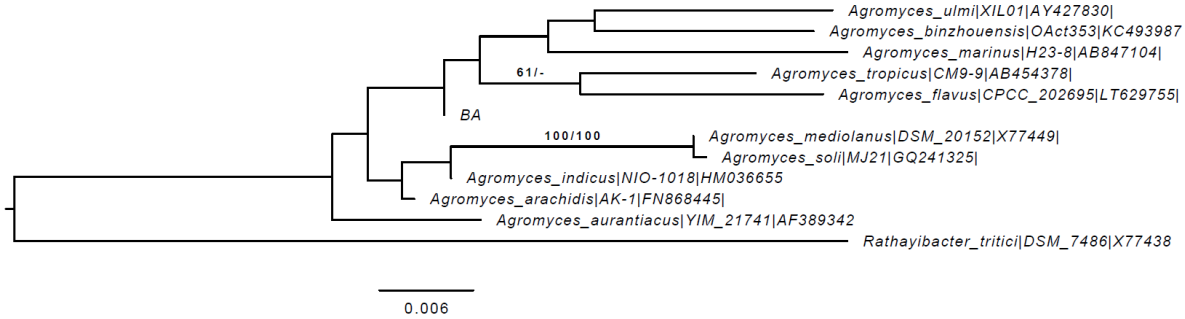


Figure 3-7 Maximum-likelihood phylogenetic tree based on full-length 16S rRNA gene sequence of BA. Numbers above the branches are bootstrap support values greater than 60% for maximum-likelihood (left) and maximum-parsimony (right) method. Bar, 0.006.

➢ Phenotype

- Physiology

Both strains grew well at different pH and temperatures up to 40 °C (Figure 3-8). They both had NaCl tolerance up to 5%. The ability to utilise different carbon sources in NS44ZA and its type strain *S. keddieii*, were different for two carbohydrates. Raffinose and inositol were used by *S. keddieii* but not NS44ZA. In contrast, strain NS44ZA showed better growth with rhamnose as carbone source (Figure S10). Strain BA utilised all carbon sources except cellulose (Figure S11). BA showed better growth than the strain *A. indicus* in the utilisation of mannose, fructose and rhamnose.

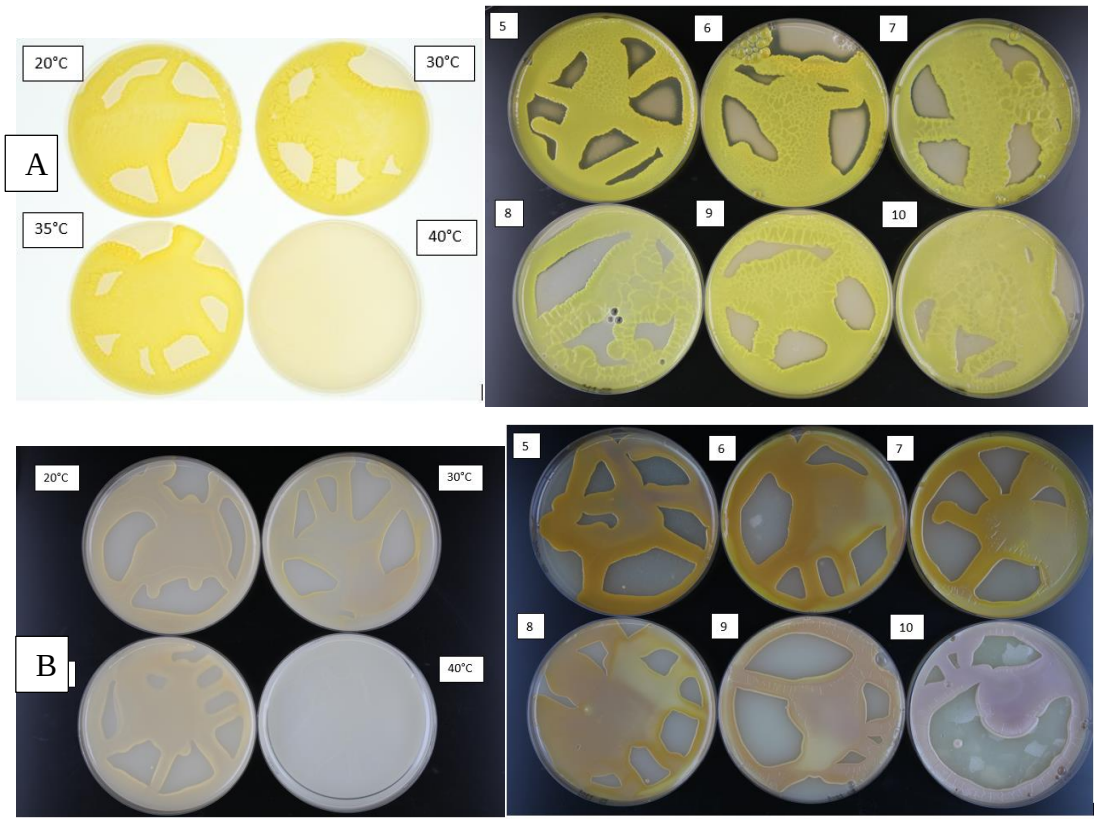


Figure 3-8 Optimum temperature and pH for strains A) NS44ZA and B) BA

- Morphology

Colonies of both strains were smooth and convex. NS44ZA showed good growth on GYM, ISP 2, 4 and 5 but sparse growth in ISP 3 and 7. BA grew well on GYM but weak growth on ISP 2-7. No soluble pigments were seen in both strains (Figure 3-9).

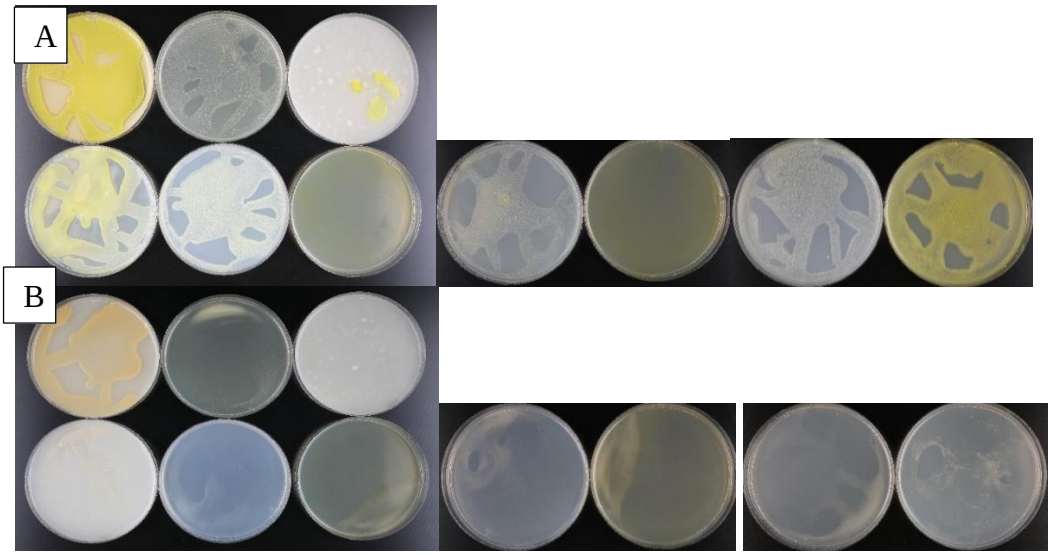


Figure 3-9 ISP and SSM plates of strains A) NS44ZA and B) BA
(left) A)GYM, B)ISP2, C)ISP3, D)ISP4, E)ISP5, F)ISP7 (right) A)ISP6, B)ISP7, C)SSM+, D)SSM-

- Biochemistry

Enyzme activity in strains NS44ZA and *S. keddieii* were mostly similar, the differences were observed in α-mannosidase and ß-glucosamidase that were active in *S. keddieii* and not in NS44ZA and the ability of NS44ZA in hydrolysing gelatin and reducing nitrate (Figure 3-10). There were no significant differences between BA and *A.indicus.* α-galactosidase was quite strong in *A.indicus* and was not produced by BA. The opposite result was in ß-galactosidase. Pyrazinamidase and esculin (ß-glucosidase) activity were stronger in *A.indicus* (Figure 3-11).

Figure 3-10 Enzyme activity of NS44ZA and *S. keddieii*

Figure 3-11 Enzyme activity of BA and *A.indicus*

➢ Chemotaxonomy

The cell-wall of NS44ZA contained MK-9(H_4) as major menaquinone and leucine, alanine, glutamic acid and lysine as amino acids. Galactose, rhamnose and ribose were detected as cell wall sugars. In both NS44ZA and *S. keddieii* diphosphatidylglycerol (DPG) and phosphatidylinositol-mannoside (PIM) were found as main polar lipids. In NS44ZA phosphatidylglycerol (PG), three unknown phospholipids, three unknown lipids and one unknown glycolipid were detected. The detected sugars in BA cell-wall were galactose, glucose and ribose. However, the detected sugars in *A.indicus* were galactose and xylose. The cell wall diagnostic amino acid was 2,4-diaminobutyric acid. Diphosphatidylglycerol (DPG) and phosphatidylglycerol (PG) were found to be the main phospholipids. One unknown glycolipid and one unknown lipid were also detected in BA (Figure 3-23). The major fatty acids in strain NS44ZA consisted of

anteiso-C15:0 (50%), *iso*-C16:0 (16%) and C16:0 (15%), which were different from type strain *S. keddieii.* In *S. keddieii*, the main fatty acids were C16:0 (53%) and *anteiso*-C15:0 (11%). The major fatty acids in strain BA were *anteiso*-C15:0 (39%), *anteiso*-C17:0 (32%) and C16:0 (20%). In *A.indicus* C16:0 (38%) was the main fatty acid and after that *anteiso*-C15:0 (28%) and *anteiso*-C17:0 (22%). The differences in fatty acids are shown in Table 3-10.

Table 3-10 Cellular fatty acid composition of strains NS44ZA, BA and their type strains

Fatty acid	**BA**	**DSM 26681**	**NS44ZA**	**DSM 10542**
		A. indicus		*S. keddieii*
saturated straight chain				
C14:0			6.1	5.8
C15:0			1.51	0.5
C16:0	2.56		15.01	53.3
C17:0				0.5
Saturated branched				
iso-C14:0	1.76	1.95	2.9	1.1
iso-C15:0	3.32	6.29	0.99	3.2
iso-C16:0	20.72	38.02	16.24	5.4
iso-C17:0		2.09		
anteiso-C15:0	39.63	28.76	50.76	11.4
anteiso-C17:0	32.01	22.89	6.5	2.4

3.1.1.3 4NS15 *Kibdelosporangium persicum*

- Ecology

Isolate 4NS15 was isolated from a desert located in the city Kerman, Iran.

- Genotype

The strain showed 16S rRNA gene sequence similarity values of 98.9 % with the type strains of *Kibdelosporangium aridum* subsp. *aridum*, *Kibdelosporangium phytohabitans*, *Kibdelosporangium philippinense* and 98.6 % with the type strain *Kibdelosporangium aridum* subsp. *largum*, respectively (Table 3-11). Based on TYGS server phylogenetic tree, strain 4NS15 with NCBI accession number MN94780 had higher similarity to *Kibdelosporangium metalli* and *philippiense* (Figure 3-12).

Table 3-11 Complete 16S rRNA gene sequencing results of 4NS15

Strain	Type strains	Similarity based on complete 16S rRNA gene	DSM code
4NS15 DSM 10728 *K.persicum*	*K.aridum* subsp. *aridum*	98.9 %	DSM 43828
	K.aridum subsp. *largum*	98.6 %	DSM 44150
	K.philippinense	98.6 %	DSM 44226
	k.phytohabitans	98.6 %	DSM 104448

Whole-genome sequencing was achieved using PacBio-sequencing by GMAK, braunschweig. The data analysis was done by Dr. Nestor Zaburannyi in HIPS. The whole-genome shotgun project was deposited at DDBJ/ENA/GenBank under the accession JAAATY000000000. Isolate 4NS15 had a genome size of 10.35 Mbp with a G+C content of 68.1 mol %. Whole-genome phylogeny was generated using the TYGS server (Figure 3-13). The dDDH value between strain 4NS15 and its closest relative *K. aridum* was 29.8% (formula d4). The calculated difference of the GC content between both strains was 2.0%.

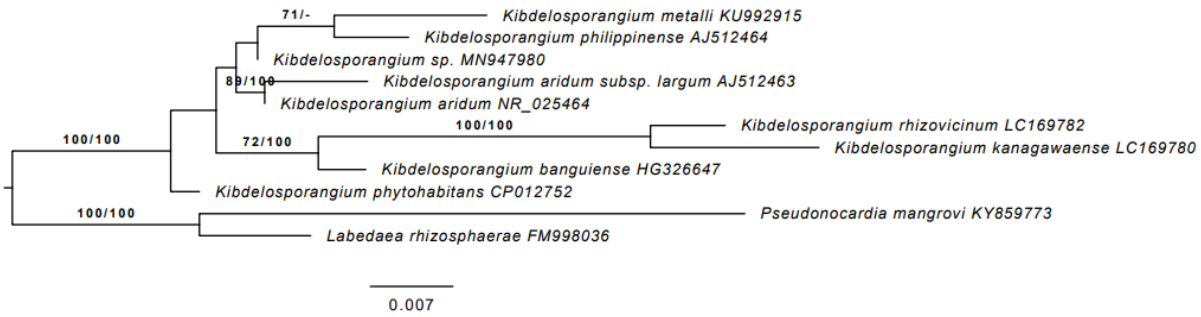


Figure 3-12 Maximum-likelihood phylogenetic tree based on full-length 16S rRNA gene sequence of 4NS15 Numbers above the branches are bootstrap support values greater than 60% for maximum-likelihood (left) and maximum-parsimony (right) method. Bar, 0.007

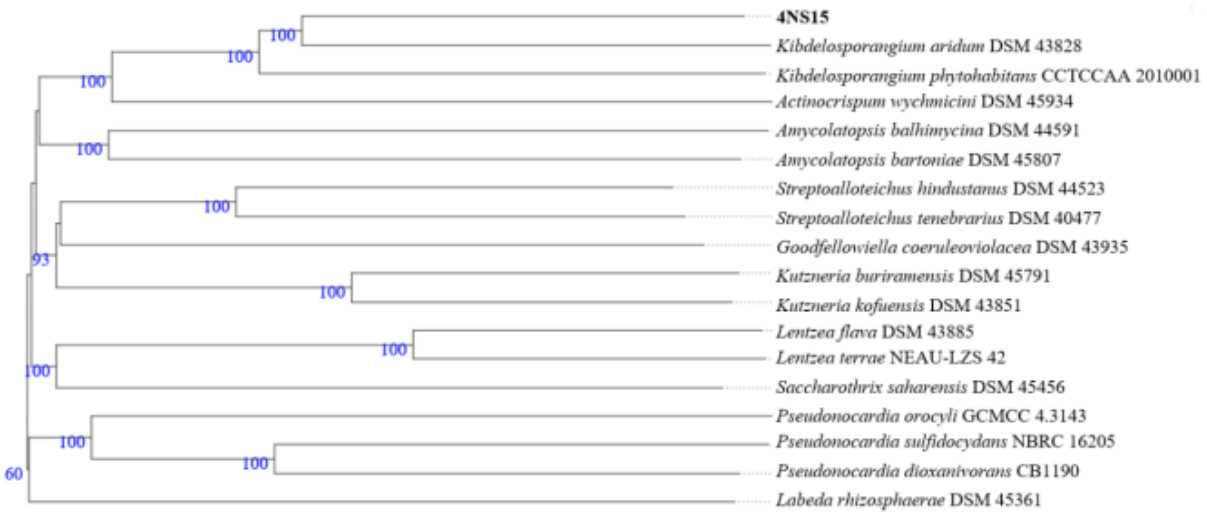


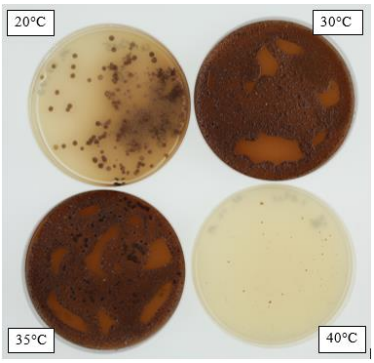
Figure 3-13 Whole-genome sequence tree generated with the TYGS web server for strain 4NS15 and closely related species

MALDI-TOF analysis was done in DSMZ and the results confirmed that *K. aridum* subsp. *aridum* and *K. aridum* subsp. *largum* had the highest similarity to the strain 4NS15 (Figure S14).

➢ Phenotype

- Physiology

Optimal growth was observed on GYM at a temperature of 35 °C, pH 7.5 and in presence of NaCl concentration up to 2.5% (w/v). D-arabinose, xylose, rhamnose and mannose were utilised as sole carbon sources, whereas D-raffinose and cellulose were not used as a carbon source (Figure 3-14).



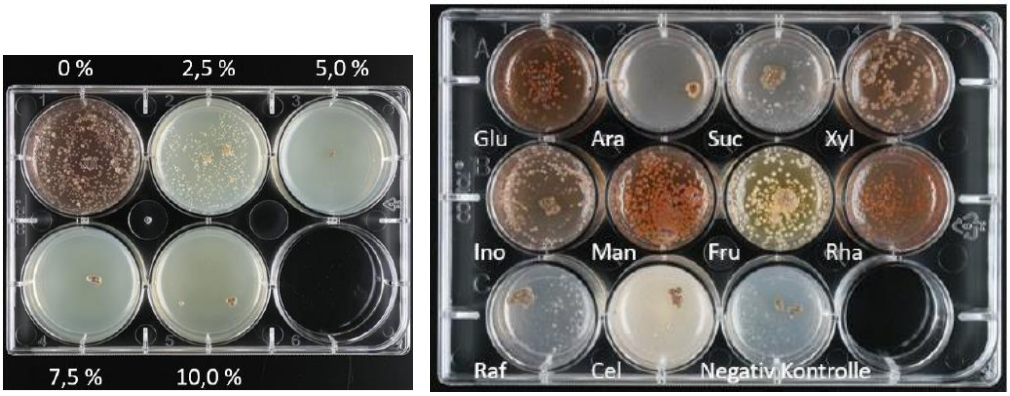


Figure 3-14 Results of temperature, pH, salt and carbon utilisation of strain 4NS15

- Morphology

Scanning electron microscopy of strain 4NS15 from ISP2 medium after 10 days showed branched substrate mycelium, without sporangium-like (pseudosporangia) structures (Figure 3-15). The black red substrate mycelium and oxide red (3007) diffusible pigments were produced on GYM, ISP2 and ISP7 (Figure S15).

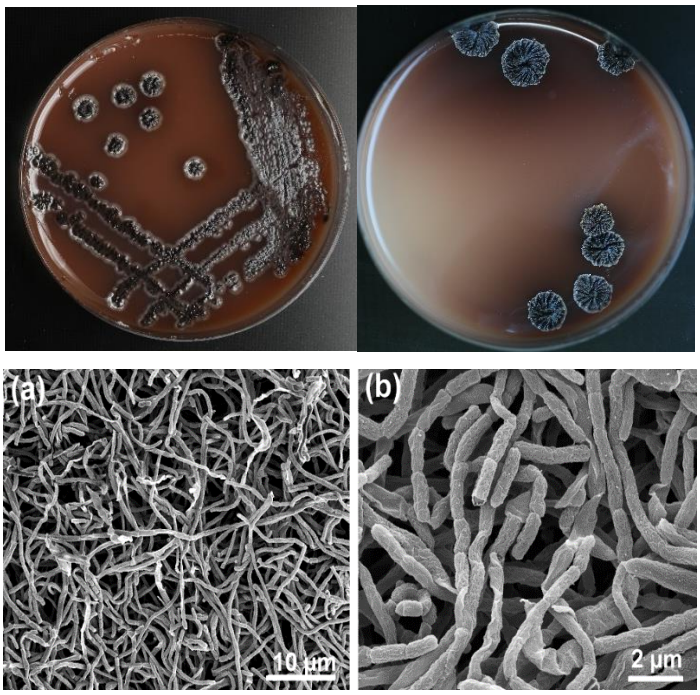


Figure 3-15 Strain 4NS15 on Gym plates (up), Scanning electron micrographs (down). a) scale 10 µm, b) scale 2 µm

- Biochemistry

Strain 4NS15 possessed substantial enzymatic activities for β-galactosidase, α-glucosidase and gelatin liquefaction but no urease activity. Esculin and nitrate reduction

were negative (Figure S16). Differences in API ZYM and API Coryne are shown in Table 3-12.

Table 3-12 Differences in enzymic activities

Enzymic activities	**4NS15**	**DSM43828^T** ***K. aridum***	**DSM44226 ^{T}** ***K. philippinense***	**DSM104448 ^{T}** ***K. phytohabitans***
Naphthol-AS-BI-phosphohydrolase	+	-	+	+
β-Galactosidase	+	+	+	-
α-Glucosidase	+	-	+	+
N-acetyl-β-Glucosaminidase	-	+	+	+
α-Mannosidase	-	+	+	+
Pyrrolidonyl arylamidase	+	+	+	-
Urease	-	+	+	+

Note: + Positive or present; - negative or absent. All the data were obtained from this study under identical growth conditions

- Chemotaxonomy

The cell wall contained *meso*-diaminopimelic acid and the whole-cell sugars were arabinose, galactose, glucose and ribose. The quinone pattern consisted of major MK-9(H_4) and minor amounts of MK-9(H_2). The phospholipid profile contained diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylhydroxyethanolamine (PE-OH), aminolipid and glycoaminolipid (Figure S17). Major cellular fatty acids pattern of 4NS15 consisted of C16:0 (25%), *iso*-C16:0 (25%) and *iso*-C15:0 (16%), which was similar to type strain *K.aridum.* The differences between strain 4NS15 fatty acid pattern and other type strains are shown in Table 3-13.

Table 3-13 Cellular fatty acid pattern of the strain 4NS15 and related species

Fatty acid	**4NS15**	**DSM 43828^T**	**DSM 44226 ^{T}**	**DSM 104448 ^{T}**
		K.aridum	*K.philippinense*	*K.phytohabitans*
saturated straight chain				
C14:0	1.4	2.2	2.9	3
C15:0	3.2	0.6		
C16:0	25.6	8.9	4.4	4.7
C17:0	6.6	0.5		
C18:0	1.3			
Saturated branched				

iso-C14:0		2.8	1.6	1.8
iso-C15:0	16.8	12.7	16.1	11.3
iso-C16:0	25	22.8	13.3	19.9
iso-C17:0		2.1	1.4	2
anteiso-C15:0	8.4	6.7	5.4	2.4
anteiso-C17:0	7.1	9.9	4.7	4.2
cy C17:0			0.7	1.2
iso-C18:0		0.4		
Unsaturated				
c/t C16:1ω7		4.5	16.5	16.3
iso-C16:1ω8		0.7	0.9	7.7
iso-C17:1ω8C	1.8	5.2	10.3	0.3
C17:1ω6C		5.3	8.4	12.6
C18:1ω5		3	3.3	2.5

- Fermentation and compound isolation

4NS15 had high activity against *M. luteus*, *S. aureus*, *E. coli TolC* and poor activity against *M. hiemalis* and *M. smegmatis.* RP-HPLC fractionation coupled to HR-ESI-MS of the extracts showed five compounds with 14 units difference in the active area of strain 4NS15 chromatogram (Figure 3-16).

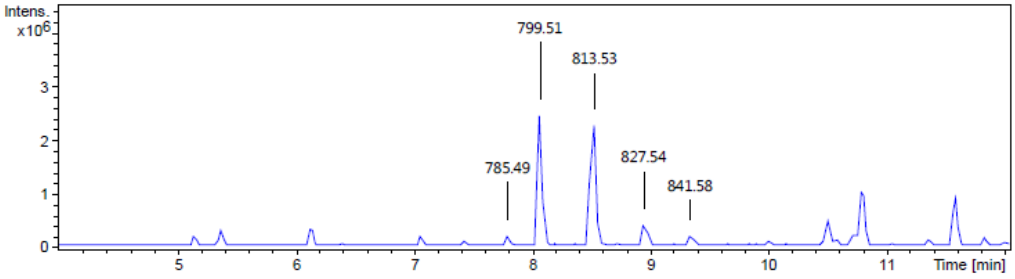


Figure 3-16 High-resolution electrospray ionization-mass spectrometry (HR-ESI-MS) total ion current (TIC) of the 4NS15 crude extract with five peaks correlated to antibacterial activity

The best medium, volume and day of harvesting were optimized. 10L of 5294 metabolite medium were harvested after 13 days (2L flasks with 1L of metabolite medium). The pellet and supernatant were separated. 5% XAD were added to the supernatant and left shaking for 24 hours. The XAD resin from the supernatant was collected by sieving, washed by flushing with distilled water and then extracted three

times with methanol. 843 gram of cell pellet were extracted with acetone. Acetone was added to cell pellet, sonicated, filtered and the extract was evaporated in a rotary evaporator. After HR-ESI-MS analysis of 4NS15, the five targeted compounds were selected for isolation. 20 µl aliquots of the extracts were injected onto an analytical size luna5µm C18(250×4.6 mm) fitted with a guard-column. The compounds were separated by multi gradient elution using water with 0.1% formic acid as solvent A and acetonitrile with 0.1% formic acid as solvent B at a flow rate of 1 ml/minute. The optimal gradient determined was from 5 to 41% solvent B in 17 minutes, from 41% to 61% of B in 20 min and from 61% to 95% of B in 2 minutes with a 6- minute hold at 95% to clean the column. MS^2 patterns have revealed that the compounds belong to one family. Among five compounds, fraction 3 (16.5mg) with ion *m/z* 813.53 corresponding to $(M+H)^+$ was isolated and purified (Table 3-14). Structural elucidation was started by Dr. Emilia Oueis in HIPS and currently it is undergoing by Dr. Lena Keller in HIPS.

Table 3-14 Isolated compounds and their weight

Fraction	Molecular weight	Pure compound weight
1	785.49	1.19 mg
2	799.51	13.3 mg
3	813.53	16.5 mg
4	827.54	3.4 mg
5	841.58	1.14 mg

The pure compound of fraction 3 inhibited the growth of A549 and MCF-7 cell lines with the IC_{50} 0.85 and 0.5 ng/ml respectively. Inhibition of the pure isolated compound on 8 cell lines were shown in Table 3-15.

Table 3-15 Cytotoxic assay ng/ml

Pure Compound	L929 Mouse fibroblast	KB3.1 Cervix carcinoma	A549 Human lung carcinoma	PC-3 Human prostate cancer	MCF-7 Human breast adenocarcinoma	A431 Squamous carcinoma	SKOV-3 Ovarian carcinoma	HUVEC Stem cells
Fr3 4NS15	3.4	1.43	0.85	2.9	0.5	2.1	2.7	750
Epo B control	0.65	0.075	0.038	0.044	0.065	0.03	0.17	0.12

3.1.1.4 Ongoing projects

Four strains NS40, NS37, NS25 and NS59 were chosen based on their taxonomy classification for additional analysis. All showed some antibacterial activity and beside polyphasic taxonomy, their crude extracts were screened for producing secondary metabolites.

NS25 and NS59

- Ecology

NS59 isolated from Isfahan desert soil and NS25 isolated from beach sand in Qeshm island.

- Genotype

16S rRNA gene sequencing results of strain NS59 showed 98% similarity to *Streptomyces specialis* DSM 41924 although from morphological aspects, they were not similar. The phylogenetic tree generated using GGDC showed NS59 with low likelihood is similar to *Streptomyces avicenniae* and *Streptomyces hainanensis* (Figure 3-17). NS25 showed 99.37% 16S rRNA gene sequencing similarity to strain *Actinomadura deserti*. Based on 16S rRNA gene sequences phylogenetic tree, NS25 showed higher similarity to *Actinomadura rifamycini* (Figure 3-18).

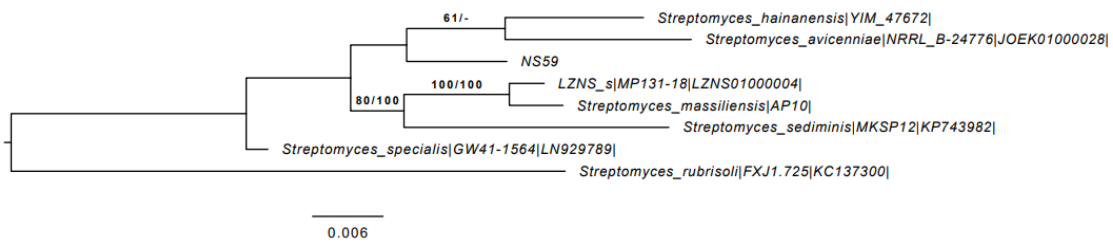


Figure 3-17 Maximum-likelihood phylogenetic tree based on full-length 16S rRNA gene sequence of NS59. Numbers above the branches are bootstrap support values greater than 60% for maximum-likelihood (left) and maximum-parsimony (right) method. Bar, 0.006.

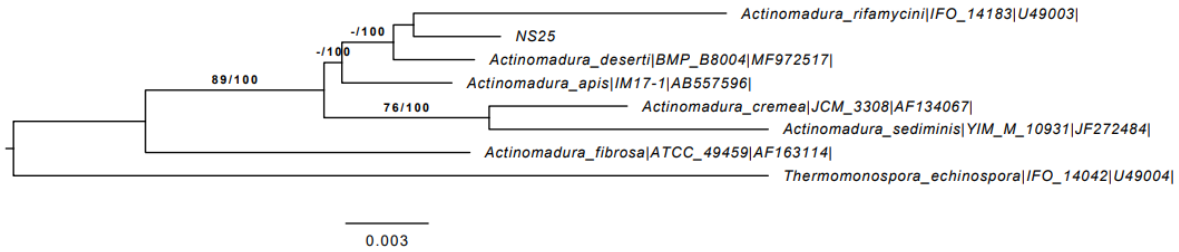


Figure 3-18 Maximum-likelihood phylogenetic tree based on full-length 16S rRNA gene sequence of NS25. Numbers above the branches are bootstrap support values greater than 60% for maximum-likelihood (left) and maximum-parsimony (right) method. Bar, 0.003.

➢ Phenotype

- Physiology

The physiological characterization was investigated just for strain NS59. The optimum growth was observed at 35 °C in absence of salt. The carbohydrate utilisation was observed sparsely in all carbon sources (Figure S18).

- Morphology

Both strains grew slowly on 5336 and GYM plates. The colonies showed different morphology compared to the most similar type strains that are shown in Figure 3-19. The colonies of NS59 appeared after 10 days and grew sparsely on ISP plates. No pigment formation was observed in ISP plates for both strains (Figure S19).

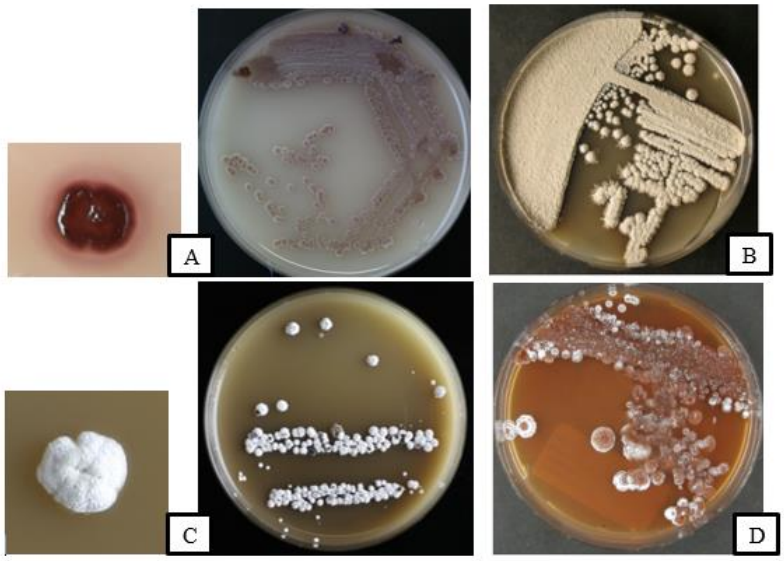


Figure 3-19 The comparison between two strains NS59 and NS25 with the most similar type strains (A= NS59 on GYM plates; B= *Streptomyces specialis*; C= NS25; D= *Actinomadura deserti*; the pictures B and D were taken from DSMZ catalogue of microorganisms)

- Screening for secondary metabolites

NS59 showed strong activity against Gram-positive bacteria and moderate activity against fungi. NS25 showed high activity against *Staphylococcus aureus* Newman and *Bacillus subtilis* DSM 10^T and good activity against Gram-negative bacteria like *E.coli* TolC. The micro-fractionation was performed in 96-well plates to target the active compounds of the crude extracts of both strains. The HPLC fractionation experiments resulted in four fractions that inhibited the growth of *S. aureus* Newman in the strain NS25. Later the crude extract analysed using HR-ESI-MS and a family of compounds detected with the main protonated ion *m/z* 254.06 $(M+H)^+$. The UV/Vis spectrum shows absorption maxima at 248 and 302 nm (Figure S20). The mass spectra of crude extract of the strain NS59 in retention time 14.5-25 minutes showed ion at *m/z* 251.20 $(M+H)^+$. The UV/Vis spectrum showed absorption maxima at 268 nm (Figure S21).

NS40 and NS37

- Ecology

Isolate NS40 was isolated from the soil around the root of plant Astragalus in the city of Yasooj and NS37 isolated from the sand in Boushehr, Iran.

- Genotype

The strain NS40 showed 16S rRNA gene sequence similarity values of 98.55% with three type strains of *Streptomyces badius*, *Streptomyces sindenensis*, *Streptomyces parvus.* Although in phylogenetic tree conducted from 16S rRNA gene sequencing, these three type strains classify in different clade but they were used for comparison in polyphasic taxonomy based on the EzTaxon database (Table 3-16)(Figure 3-20).

Strain NS37 showed 99.58% similarity to *Nocardiopsis umidischolae* DSM 44362 and 99.38% to *Nocardiopsis tropica* DSM 44381. According to phylogenetic tree analysis, NS37 showed higher similarity to *N. tropica* (Figure 3-21).

Table 3-16 Complete 16S rRNA gene sequencing results of NS40 and NS37

Strain	Type strains	Similarity based on 16S rRNA gene	DSM code
NS40 CIP 111700	*Streptomyces badius*	98.55%	DSM 40139
	Streptomyces sindenensis	98.55%	DSM 40255
	Streptomyces parvus	98.55%	DSM 40348
NS37 CIP 111703	*Nocardiopsis umidischolae*	99.58%	DSM 44362
	Nocardiopsis tropica	99.38%	DSM 44381

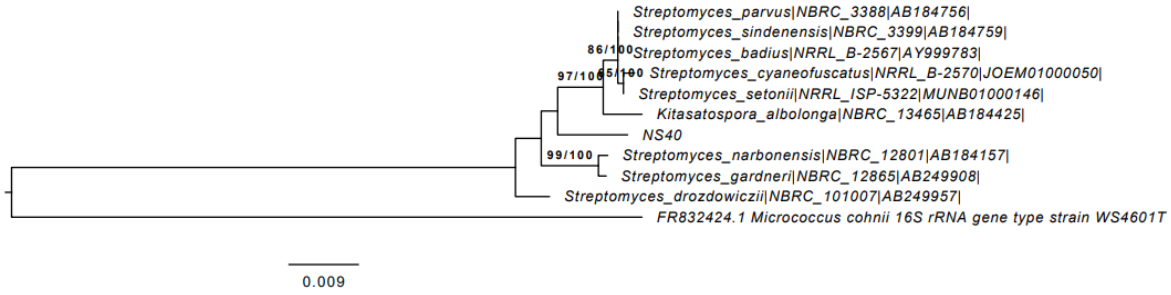


Figure 3-20 Maximum-likelihood phylogenetic tree based on full-length 16S rRNA gene sequence of NS40. Numbers above the branches are bootstrap support values greater than 60% for maximum-likelihood (left) and maximum-parsimony (right) method. Bar, 0.009.

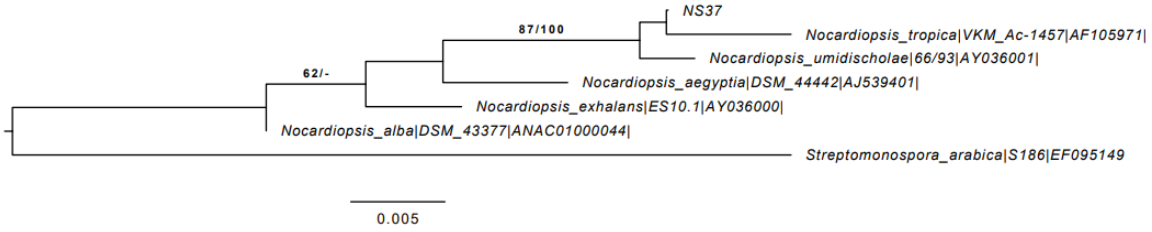


Figure 3-21 Maximum-likelihood phylogenetic tree based on full-length 16S rRNA gene sequence of NS37. Numbers above the branches are bootstrap support values greater than 60% for maximum-likelihood (left) and maximum-parsimony (right) method. Bar, 0.005.

The DNA of both strains and the closest type strains (Table 3-16) were sent to Dr.Kämpfer for DDH analysis. The results were 25% between the strain NS40 and *S.badius* and 65.4% between NS37 and *N.umidischolae*, that was lower than the threshold value (70%) for identification of new species. The MLST analysis were conducted for both strains with three house keeping genes (trpB, gyrB and atpD). Strain

NS40 was found in a different clade based on 16S rRNA gene sequencing and from MLSA results (Figure 3-22).

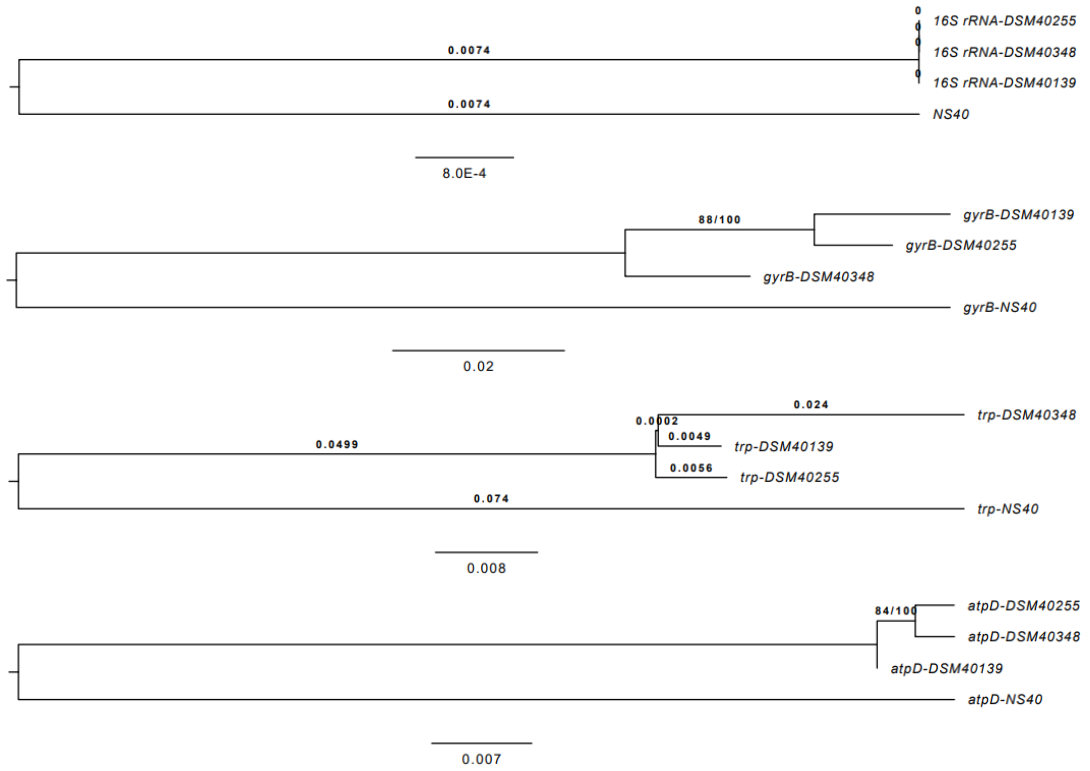


Figure 3-22 Maximum-likelihood and parsimony tree of strain NS40 and close type strains based on partial sequences of the three house-keeping genes gyrB, trpB and atpD

Based on 16S rRNA gene sequencing, strain NS37 was closely related to *N. tropica* DSM 44381^T rather than *N. umidischolae* DSM 44632^T. The MLSA results showed that for gyrB gene, NS37 had higher similarity to *Nocardiopsis alba* (the sequences obtained from NCBI). The phylogenetic tree of trpB gene sequencing showed that NS37 shared same clade with *N. umidischolae*. The MLSA database lack sequences for atpD from *Nocardiopsis*, for that reason, *Streptomyces* MM98 was used in atpD phylogenetic tree and NS37 showed higher similarity in this gene to *Streptomyces* over *Nocardiopsis* (Figure 3-23).

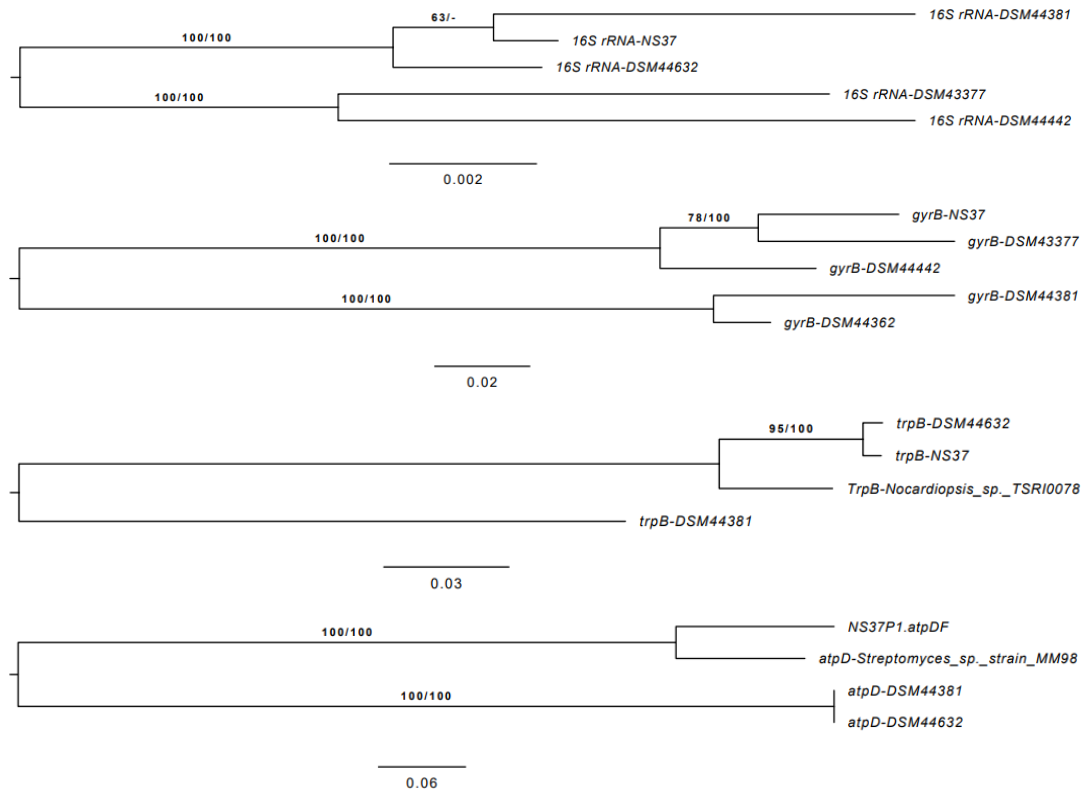


Figure 3-23 Maximum-likelihood and parsimony tree of strain NS37 and close type strains based on partial sequences of the three house-keeping genes gyrB, trpB and atpD.

The MALDI-TOF dendrogram indicated that strain NS37 is similar to *N. umidischolae* with no distance level (Figure S22).

➢ Phenotype

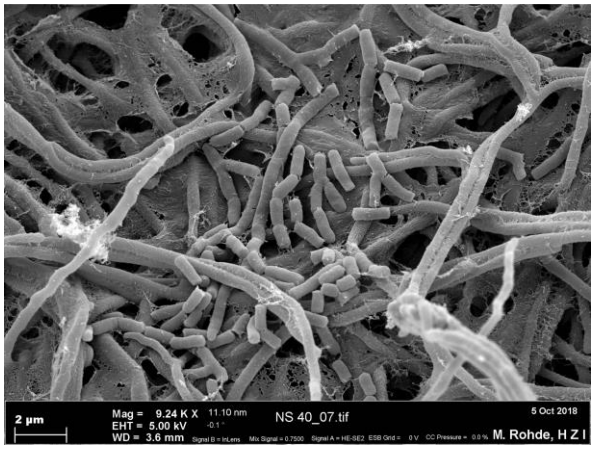
- Physiology

The NaCl concentration that the strain NS40 could tolerate was 5% and for the strain NS37 was up to 10% (w/v). D-raffinose, cellulose and inositol were not used as a carbon source in NS40. NS37 could utilise different carbon sources except inositol and cellulose (Figure S23).

- Morphology

Scanning electron microscopy of strain NS40 from ISP3 medium after 10 days showed long aerial mycelium with spores and NS37 has broadly branched mycelium with chains of spores (Figure 3-24).

A



B

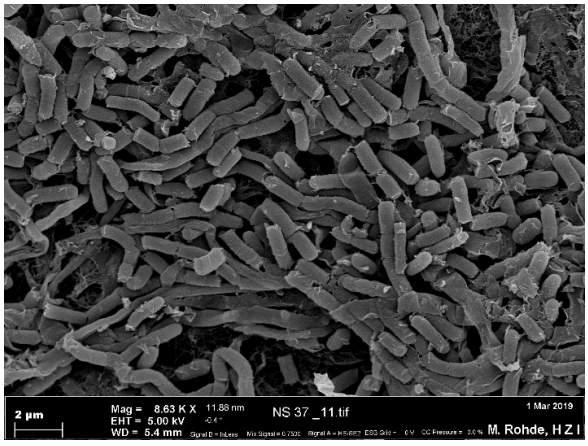


Figure 3-24 Scanning electron micrographs. A) Strain NS40, scale 2 µm, B) Strain NS37, scale 2 µm

Strain NS40 grew well in all ISP media and no pigment formation was observed for the isolate. It had higher similarity to DSM 40139^T, compared to other type strains, which formed white substrate mycelium (Figure S24). Strain NS37 had good growth in all ISP media and no pigment formation was observed for the strain. While DSM44381^T formed red soluble pigment (Figure S25).

- Biochemistry

Many enzymatic activities were similar in the strain NS40 and its type strains. Some differences observed in α-chymotrypsin. The isolate NS40 showed less activity for chymotrypsin, compare to the other type strains. Strain NS40 possessed substantial enzymatic activities for N-acetyl-β-glucosaminidase. Nitrate reduction in NS40 and DSM40255^T were similar and strong in comparison to the other type strains (Figure S26).

NS37 shared same enzyme activities with the other two type strains, except the activity of β-galactosidase and α-mannosidase, which were strong, just in the strain NS37 (Figure 3-25).

Figure 3-25 Enzyme activity of strain NS37 and related type strains

➢ Antibacterial assay of NS40

The results of MIC showed that strain NS40 had strong antifungal activity. After media optimization, the metabolite medium 5294 was chosen for fermentation. The strain cultured in 50 flasks containing 100 ml of medium 5294 in 250 ml Erlenmeyer shaking for 7 days at 28 °C. After micro-fractionation of its crude extract using HPLC and later analysing HR-ESI-MS results, the data showed multiple compounds with ions *m/z* 926.5, 910.5, 896.5 and 880.5 corresponding to $(M+H)^+$. The UV/Vis spectrum showed absorption maxima at 222, 320, 336 and 352 nm (Figure S27).

A search in the Dictionary of Natural Products (DNP), revealed that the protonated ion *m/z* 925.503504 $(M+H)^+$ matched with the known compound, Amphotericin A with the molecular formula $C_{47}H_{75}NO_{17}$. Amphotericin A produced by *Streptomyces nodosus* and known as antifungal agent (114). Some analytical scale optimizations for the separation of the mixture were unsuccessful and the project halted.

➢ Antiviral assay of NS37

The crude extract of strain NS37 that was harvested after 7 days from medium 5294 and sent to TWINCORE for antiviral assay. The results revealed that the strain had strong antiviral activity (3-20% infectivity) against hepatitis C virus (HCV). Further studies on the strain NS37 halted due to our unsuccessful cooperation.

3.1.2 Myxobacteria

Thirty-two myxobacteria were isolated from different locations in six cities of Iran and from the city Braunschweig in Germany. The list of all isolates with partial 16S rRNA sequence similarity are shown in Table 3-17.

Table 3-17 List of 32 isolated Myxobacteria

City	Code	Type of sample	Isolated Myxobacteria	Similarity 16S rRNA (Partial %)
Qeshm	NSX1,3,5,8,9,18	Sand	*Myxococcus xanthus*	99.57, 99.75, 99.57, 99.51, 99.55, 99.58
Yasooj	NSX2,4,6,46	Soil	*Myxococcus xanthus*	99.31, 99.35, 99.26, 100
Semnan	NSX7,13,20	Desert soil	*Myxococcus virescens*	99.65, 98.80, 100
Boushehr	NSX10	Sand	*Myxococcus xanthus*	100
Isfahan	NSX12,14,15,17	Soil	*Corallococcus exiguas*	98.59, 99.77, 100, 99.45
Kerman	NSX16,4NSX1	Desert soil	*Archangium gephyra*	99.88, 99.01
	NSX19	Desert soil	*Sorangium cellolosum*	98.97
	4NSX7, 4NSX10	Desert soil	*Nanocystis pussila*	99, 99.62
	4NSX9	Desert soil	*Hyalangium minutum*	99.27
	4NSX5,4NSX8,4NSX2	Desert soil	*Myxococcus flavus*	99.88, 99.76, 99.86
	4NSX4,4NSX3	Desert soil	*Myxococcus xanthus*	100, 99.89
	7NSX	Desert soil	*Myxococcus virescens*	100
Braunschweig	T4	Soil	*Corallococcus macrosporus*	100
	3WSO	Soil	*Myxococcus hansupus*	99.87

Twenty-one strains of all Myxobacteria (66%) were characterized as members of the genus *Myxococcus* and 16% of isolates were characterized as members of the genus *Corallococcus* (Figure 3-26).

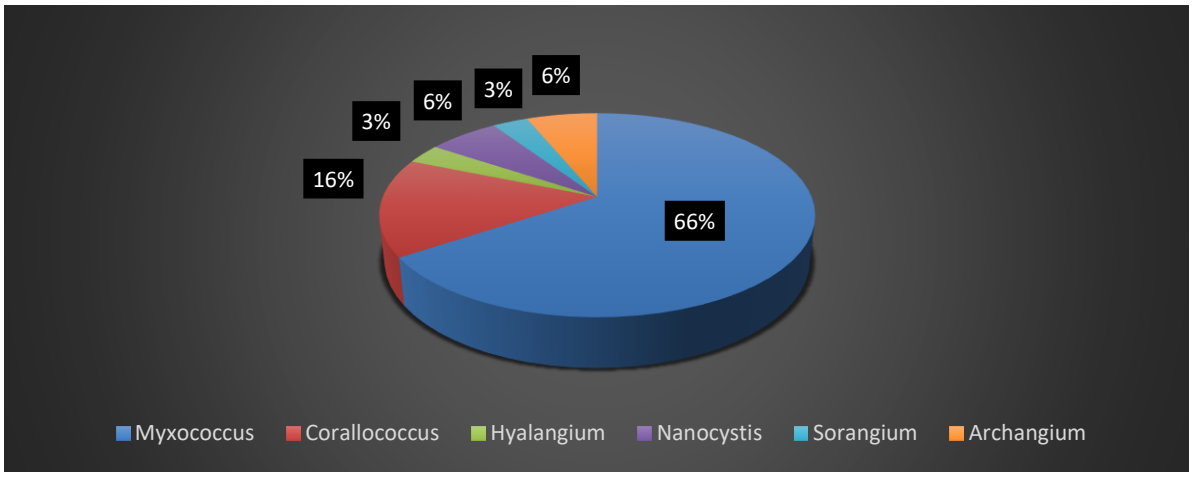


Figure 3-26 Genus-level diversity of Myxobacteria isolated from soil samples

All isolates were tested for antimicrobial activity and one strain (4NSX3) with the best activity against *E. coli* DSM 1611 was chosen for further investigation.

3.1.2.1 4NSX3

- Ecology

Strain 4NSX3 was isolated from the desert soil Kerman, Iran.

- Genotype

Based on 16S rRNA gene sequencing similarity, it was 99.89 similar to *Myxococcus xanthus*.

- Screening for secondary metabolites

Strain 4NSX3 showed strong activity against *E. coli* TolC and *E. coli* DSM 1116. The crude extract was fractionated against *E. coli* DSM 1116 and the retention time of active zone was between 29.5 and 30.5 min. In HPLC results there was a small peak representing the active area, which appeared in HR-ESI-MS chromatogram as well (Figure S28). A search in Myxobase in-house database resulted in detection of the known compound, myxovirescin C.

3.2 Snail project

From the collected snails during this study the total number of 28 Actinobacteria and 4 Myxobacteria were isolated (Table 3-18). The isolated Actinobacteria belonged to different genera, including *Streptomyces*, *Micromonospora*, *Nocardia*, *Kitasataspora* and *Nonomuraea* (Figure 3-27). The isolated Myxobacteria belonged to the genus *Myxococcus*.

Table 3-18 Isolated Actinobacteria and Myxobacteria from snails

City	Code	Genus of snail	Isolated Actinobacteria and Myxoacteria	Similarity 16S rRNA (Partial)
Mahmodabad (Rice field)	NS1	*Succinea*	*Streptomyces geysiriensis*	100
Chamestan (Forest)	NS2	*Succinea*	*Streptomyces parvulus*	99.76
Chalous (Side road)	NS3	*Succinea*	*Streptomyces hydrogenaus*	99.66
Schladen	NS4	*Planorbis*	*Micromonspora aurantiaca*	100
Isingerode (Fishing area)	NS5	*Planorbis*	*Streptomyces phaeluteigriseus*	99.58
Hanau	NSMX	*Physa*	*Myxococcus xanthus*	100
Gießen	2Fkh	*Lymnaea*	*Kitasatospora cheerisanensis*	99.67
Walluf	2Fs	*Lymnaea*	*Streptomyces luridiscabiei*	99.88
Darmstadt	3F1D	*Planorbis*	*Streptomyces anulatus*	100
Rödermark	3F1HN	*Planorbis*	*Micromonospora saelicesensis*	100
Darmstadt (Botanischergarten)	5NS1,9	*Succinea*	*Micromonospora chalcea*	99.86
Gießen		*Physa*		
Rödermark	5NS2	*Lymnaea*	*Streptomyces omiyaensis*	100
Gießen	5NS3	*Potamopyrgus*	*Micromonospora schwarzwaldensis*	99.75
Braunschweig	5NS4	*Thiaridae*	*Micromonospora citrea*	99.49
	5NS5	*Lymnaea*		99.53
	5NS6	*Lymnaea*	*Nocardia salmonicidae*	100
	5NS7	*Lymnaea*	*Nocardia ignorata*	99.69
	5NS11	*Lymnaea*	*Streptomyces paradoxus*	99.88
	5NSX1	*Lymnaea*	*Myxococcus virescas*	99.54

5NSX5	*Potamopyrgus*	*Myxococcus virescas*	100
7NS1	*Physa*	*Streptomyces violascens*	99.75
7NS3	*Physa*	*Streptomyces seoulensis*	99.2
7NS5	*Physa*	*Streptomyces speibonae*	99.6
7NS6	*Physa*	*Micromonospora echinospora*	100
7NSX	*Physa*	*Myxococcus virescas*	100
7NS4	*Lymnaea*	*Streptomyces hydrogenaus*	99.54
8NS1	*Lymnaea*	*Streptomyces drozdowiczii*	99.77
9NS1	*Potamopyrgus*	*Streptomyces hydrogenaus*	100
9NS3	*Bithynia*	*Streptomyces dijakartensis*	99.49
9NS4(3MO)	*Radix*	*Nonomuraea fastidiosa*	99.13
9NSX(3WO)	*Radix*	*Myxococcus xanthus*	99.87
9NS5	*Planorbis*	*Streptomyces yanii*	100
9NS6	*Ancylus*	*Streptomyces badius*	100
9NS7	*Planorbis*	*Streptomyces fulvissimus*	100

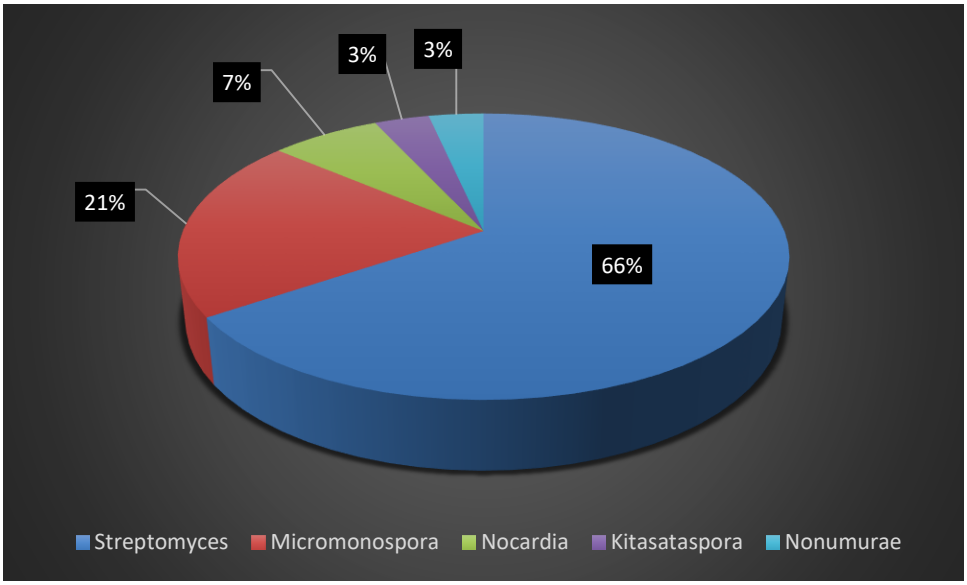


Figure 3-27 Genus-level diversity of isolated Actinobacteria from snail samples

3.2.1 Microbial community of three freshwater snails

To gain insight into the composition of the bacterial community (especially of the phylum Actinobacteria), of the three freshwater snails species *Lymnaea baltica*,

Lymnaea stagnalis and *Physa acuta*, a metagenome approach had been applied at the DSMZ, whereby amplicons of the V3 region of the 16S rRNA gene were analysed. DNA of the whole snails was extracted using the PowerSoil® DNA Isolation Kit and quantified using a nanodrop (Thermo Scientific). Amplicon sequencing of the V3 region of the 16S rRNA gene yielded 340 Mbp of raw sequence data in the form of 1,124,851 paired-end reads, with reads lengths of 2x151 bp. After amplicon analysis including: trimming, joining and removal of low-quality sequences, 894,391 full-length amplicons were obtained from, which 880,108 could be assigned to the bacterial domain by RDP Classifier. This part was done by Dr. Boyke Bunk and Dr. Katharina Huber.

The results indicated that the predominant phyla in freshwater snails of *Lymnaea baltica, Lymnaea stagnalis* and *Physa acuta,* were Tenericutes, Firmicutes and Proteobacteria, respectively. The proportation of Actinobacteria and Myxobacteria were low in all snails (less than 2%) (Figure S29). From *Lymnaea baltica* and *Lymnaea stagnalis*, two Actinobacteria were isolated, which were close related to *Streptomyces drozdowiczii* and *Streptomyces hydrogenous*. From *Physa acuta* four Actinobacteria and one Myxobacterium were isolated. Three of the Actinobacteria belonged to the genus *Streptomyces* and one belonged to the genus *Micromonospora*. The list of isolated strains and their similarity based on complete 16S rRNA gene sequencing are shown in Table 3-19.

Table 3-19 List of isolated Actinobacteria and Myxobacteria from three freshwater snails

Code	Genus of snail	Isolated Actinobacteria and Myxoacteria	Similarity 16S rRNA (Partial)
7NS1	Physa acuta	*Streptomyces violascens*	99.75
7NS3	Physa acuta	*Streptomyces seoulensis*	99.2
7NS5	Physa acuta	*Streptomyces speibonae*	99.6
7NS6	Physa acuta	*Micromonospora echinospora*	100
7NSX	Physa acuta	*Myxococcus virescas*	100
7NS4	Lymnaea stagnalis	*Streptomyces hydrogenaus*	99.54
8NS1	Lymnaea baltica	*Streptomyces drozdowiczii*	99.77

3.2.1.1 Freshwater snail *Physa acuta*

From the three mentioned freshwater snails, a *Streptomyces* (7NS3), which was isolated from *Physa acuta* was chosen for analysing the whole-genome sequence, screening for producing antibiotics and polyphasic taxonomy. The reasons of targeting this strain over the others, were having lower similarity in 16S rRNA gene sequencing and higher antimicrobial activity of its crude extract, compare to the other strains.

3.2.1.2 Strain 7NS3

- Ecology

Isolate 7NS3 was isolated from freshwater snail *Physa acuta*, which was collected from a pond (52° 6’ 42.32” N, 10° 40’ 46.20” E) in Remlingen-Semmenstedt, Germany in June 2019 by Dr. Joachim Wink.

- Genotype

Based on complete 16S rRNA gene sequencing, 7NS3 showed the highest similarity of 99.59% to *Streptomyces seoulensis* NRRL B-24310^T in the EzTaxon database. Phylogenetic tree results using GGDC server were in line with 16S rRNA gene sequencing result and showed the strain 7NS3 had the highest similarity to *S. seoulensis* (Figure 3-28).

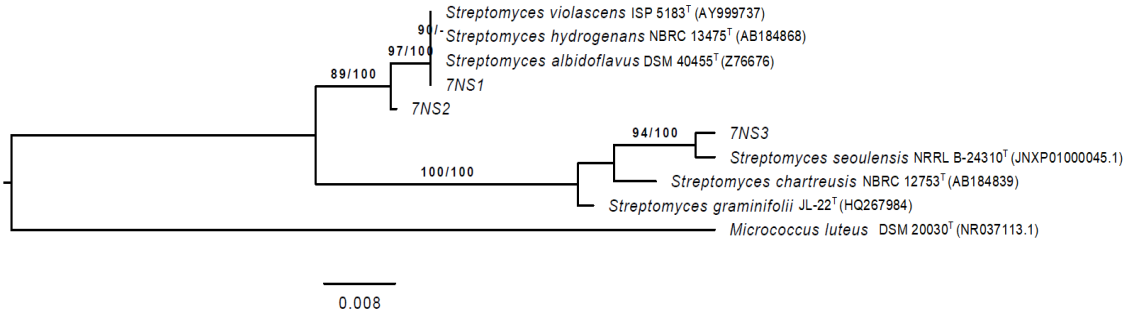


Figure 3-28 Maximum-likelihood phylogenetic tree based on full-length 16S rRNA gene sequence of 7NS3. Numbers above the branches are bootstrap support values greater than 60% for maximum-likelihood (left) and maximum-parsimony (right) method. Bar, 0.008.

The draft genome sequence of 7NS3 was obtained using Illumina whole-genome sequencing performed in DSMZ. The genome was assembled to 49 quality-controlled contigs (> 450 bp) summing up to a total genome size of 6.85 Mbp with a GC content

of 71.6%. Digital DNA-DNA Hybridization (dDDH) of *Streptomyces* sp. 7NS3 and *S. seoulensis* NRRL B-24310^T showed the value of 29.5%, which was below the threshold of 70%. 7NS3 represented a novel *Streptomyces* species, which was deposited as *Streptomyces* sp. NCCB100804 and DSM 110735.

- Phenotype

- Physiology

Strain 7NS3 grew at pH 6,7 and 8 but the optimum growth was observed at pH 7. Strain 7NS3 tolerated up to 5% NaCl and had the ability to utilise different carbon sources (Figure S30).

- Morphology

Scanning electron microscopy of strain 7NS3 from ISP3 medium after 10 days showed long-chain of spores with smooth surfaces (Figure 3-29). The gray substrate mycelium and aerial mycelium were observed in all ISP plates and they formed no pigments on melanin plates (Figure S31).

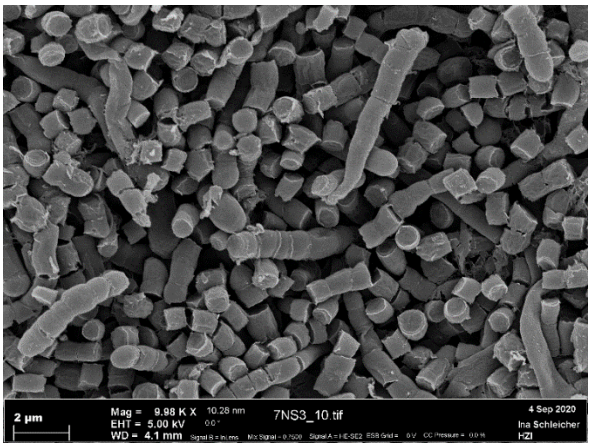


Figure 3-29 Scanning electron micrographs, scale 2 µm

- Biochemistry

Strain 7NS3 possessed substantial enzymatic activities for alkaline phosphatase, leucine arylamidase, acid phosphatase and α-glucosidase. Esculin and nitrate reduction were negative (Figure 3-30).

Figure 3-30 Enzyme activity of strain 7NS3

- Chemotaxonomy

The cell wall contains LL-diaminopimelic acid and the whole-cell sugars were mannose, glucose and ribose. The quinone pattern consisted of major MK-9(H_4) and MK-9(H_6). The present phospholipids were phosphatidyl-N-methylethanolamine (PME), diphosphatidylglycerol (DPG), glucosamine (GAL) and phosphatidylinositol (PI) (Figure S32). Major cellular fatty acids pattern of strain 7NS3 consisted of C15:0a (28.9%), C16:0i (21.6%) and C16:0 (9%).

- Metabolite profiling

The MIC results from the crude extract (1mg/ml) of strain 7NS3 had the highest activity against *S. aureus* (Newman) and *B. subtilis* DSM 10^T with an MIC value of 8.3 µg/ml and 0.52 µg/ml, respectively.

The 7NS3 crude extract was fractionated by HPLC to determine the peak-related activities. The micro-fractionation resulted in four fractions that inhibited the growth of B. subtilis DSM 10^T and two fractions inhibiting *S. aureus* Newman. In order to identify the associated target masses of the detected active fractions, the crude extract was analysed using HR-ESI-MS. The four peaks, which were correlated to activity in the HPLC chromatogram, were detected in the HR-ESI-MS total ion current (TIC) and named compounds 1 to 4 (Figure S33). The mass spectra of compound 1 with the retention time (t_R) = 10.39 min showed ions at a mass-to-charge ratio (*m/z*) of 289.0857, corresponding to $(M+H)^+$. The UV/Vis absorption bands were located at 232 nm, 380 nm and 458 nm. The second compound, with a chromatographic retention time of 10.49 min, presented mass spectra with ions *m/z* 307.0966, *m/z* 329.0785 and *m/z* 289.0859 corresponding to $(M+H)^+$, $(M+Na)^+$ and $(MH^+ - H2O)$, respectively. The third molecule, with t_R= 11.49 min, showed ions *m/z* 309.1118, *m/z* 331.0939 and *m/z* 291.1014 corresponding to $(M+H)^+$, $(M+Na)^+$ and $(MH^+ - H2O)$, respectively. The suggested formulas of $C_{19}H_{14}O_4$ and $C_{19}H_{16}O_4$ were obtained for compounds 3 and 4

by the SmartFormula™ algorithm in Compass software from Bruker. A search in the Dictionary of Natural Products (DNP) database revealed that the protonated ion *m/z* 309.1118 $(M+H)^+$ matched with the known compound, emycin A. Emycin A has a molecular weight of 308.3436 g mol^{-1} (*m/z* 309.3436 $(M+H)^+$) and a formula of $C_{19}H_{16}O_4$. The UV/Vis spectrum showed absorption maxima at 217 nm, 260 nm and 402 nm. The characteristic UV/Vis absorption bands at 220 nm, 260 nm and 402 nm of compound 3 matched with those of emycin A. The chromatogram pattern of compounds 1 and 2 indicated the structural isomers of the main compound. From the UV bands and mass similarity, it can be concluded that compound 3 has a double bond compared to compound 2. Compound 4 with a t_R of 15.39 min presented a mass spectra with ions *m/z* 255.2319 and *m/z* 256.2635 corresponding to $(M+H)^+$ that matched with hexadecenoic acid in the in-house Myxobase database. This fatty acid with the molecular formula of $C_{16}H_{30}O_2$ had antibacterial activity against Gram-positive bacteria.

- BGC identification and antiSMASH output

The genome sequence was analysed with the bioinformatic tool antiSMASH 5.0. A total of 12 BGCs were detected, whereby six of them matched known clusters for alkylresorcinol, scabichelin, informatipeptin, geosmin, albaflavenone and ectoine with 100% similarity. One BGCs showed >80% similarity to a BGC encoding a potential spore pigment. Three BGCs showed >60% similarity to clusters encoding desferrioxamin, melanin and hopene. The remaining BGCs with similarities <50% were predicted to encode two nonribosomal peptides, one polyketide, one polyketide-nonribosomal peptide hybrid, one thiopeptide, one siderophore, one lassopeptide, one terpene and one bacteriocin (Figure S34). The BGC identification and antiSMASH analysis were performed by Dr. Yvonne Mast. Based on antiSMASH analysis results, the strain 7NS3 harbored a potential angucycline-like BGC, which is suggested to code for the emycin A-like substance. To prove the functionality of the identified BGC and to elucidate the chemical structures of the identified substances, further experiments are needed.

4 Discussion

Identification of less than 1% of the existing microorganisms until date implies the presence of a large number of microorganisms in natural environments that are yet to be fully explored (81). Microbial natural products are promising sources for drug discovery, although access to new scaffolds with strong activity have become difficult over past few decades. That is mostly because of rediscovery issues, challenges of isolating new producers and low concentration of the producer's secondary metabolites (115). Among prokaryotes, filamentous actinomycetes, the myxobacteria, the pseudomonads and cyanobacteria have the potential to produce many diverse metabolites (116). Soil as the most significant source of actinomycetes has been explored extensively across the globe. Previously this exploration resulted in discovery of many new antibiotics but recently, rediscovery of already known species and compounds, grew the interest towards under-investigated habitats (81). Deserts, mangrove forests, medicinal plants and symbiotic association (specifically freshwater snails) are chosen as unexplored habitats within the scope of this research. Although deserts account for 20% of the landmass on the planet, they have received little attention from microbiologists (61). The most studied desert in terms of isolating Actinobacteria is the Atacama Desert in Chile (61). The strains isolated from this well-known desert belong not only to the ubiquitous genus *Streptomyces*, but also to rare or scarcely studied genera like *Actinomadura*, *Amycolatopsis*, *Nocardiopsis*, *Nonomuraea*, *Saccha- ropolyspora* and *Saccharothrix*. The most culture-independent studies of arid areas show Actinobacteria as one of the three most abundant phyla and in case of the Atacama Desert, it is the most dominant phylum (72-88%) (117).

4.1 Isolates from the desert

In order to isolate Actinobacteria and Myxobacteria from the desert, we collected three samples from two deserts in Iran. Rige jen in the border of Semnan and Isfahan provinces and Shahdad in city Kerman were the locations for sands collection. The strategy applied for routine isolation process was using 5336 medium for Actinobacteria and water agar plus *E.coli* and ST21 for Myxobacteria isolation. However, for Isfahan sand samples other strategies of pretreatment were performed to isolate rare or non-*Streptomyces*. The pretreatments included heating the soil at 60 ˚C for 30 minutes, adding chloramine T, using humic acid-vitamin agar (HV), ISP4 and ISP2 plates for the first step isolation. The research team lead by Hayakawa worked extensively

on developing different methods or various media combination in order to isolate uncommon or less studied Actinobacteria (118). *Streptomyces* is the largest and the most dominant genus in normal isolation methods. It is also important because of its ability in the production of the large number of natural antibiotics such as streptomycin, daptomycin, erythromycin, fosfomycin, lincomycin, neomycin and tetracycline (119). The normal isolation media including 5336 medium contain starch as carbon source and casein as nitrogen source that results in the good growth of *Streptomyces*. However, in this study as mentioned above, we tried to isolate non-*Streptomyces* genera by treating the sand sample. The rare actinomycetes or non-*Streptomyces* are usually regarded as strains of actinomycetes that are slow growing and difficult to isolate or culture. Currently, they have been known as highly prospective sources of bioactive compounds (119). HV agar, which was used as one of the pretreatment methods (containing soil humic acid as the sole carbon and nitrogen source), permit good growth of not only *Streptomyces* colonies, but also other actinomycetes belonging to genera *Microbispora*, *Streptosporangium*, *Micromonospora* and *Dactylosporangium* (84). The isolated bacteria from two untreated sands belonged to the genera of *Streptomyces* and *Kribbella* from Rige jen and *Kibdelosporangium* and *Saccharothrix* from Shahdad desert.

4.1.1 Description of strain 4NS15 and its secondary metabolites

Among isolates from untreated sands, the isolate 4NS15, which belonged to the genus *Kibdelosporangium* showed 16S rRNA gene sequence similarity values of 98.9 % to three type strains of *Kibdelosporangium aridum* subsp. *aridum*, *Kibdelosporangium phytohabitans* and *Kibdelosporangium philippinense* and 98.6 % to the type strain *K. aridum* subsp. *largum*. The genus *Kibdelosporangium* is considered as a rare genus, which belongs to the family Pseudonocardiaceae. *Kibdelosporangium aridum* subsp. *aridum* DSM 43828^{T} is the type species and this taxon encompasses nine validly published species (66). Members of this genus grow aerobically and their cell-wall peptidoglycan contain *meso*-diaminopimelic acid. The whole-cell sugar pattern consists of arabinose, galactose and some traces of madurose (120). This genus is famous for forming sporangium-like structures (pseudosporangia), whereas isolate 4NS15 did not have this typical characteristic. In terms of genotype the strain has a genome size of 10.35 Mbp with a G+C content of 68.1 mol% and the dDDH value between strain 4NS15 and its closest relative *K. aridum* was 29.8%, which is below threshold of 70%. In phenotypic analysis, it showed more similarity to *K. aridum* subsp. *aridum.* On the other hand, there are some significant differences in enzyme activity and the pigment formation of both strains. The

phospholipid profile of strain 4NS15 contains diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylhydroxyethanolamine, aminolipid and glycoaminolipid. The predominant menaquinone is MK-9(H4) and major fatty acids (>10 %) are C16:0, iso- C16:0 and iso- C15:0. In our recently published work, we have described the polyphasic taxonomic study of this strain (121). Based on its genotypic and phenotypic characteristics described in our work, the strain represents a novel species with the proposed name *Kibdelosporangium persicum* sp. nov. and has been deposited as NCCB 100701=CIP 111705=DSM 110728.

The genus *Kibdelosporangium* is known to produce novel antibiotics with glycopeptide, macrolides and polyketide structures (122). Aridicins A, B and C are three glycopeptide antibiotics produced by *K. aridum* subsp. *aridum* (123). Isolate 4NS15 showed high activity against *M. luteus*, *S. aureus*, *E. coli TolC* and poor activity against *M. hiemalis* and *M. smegmatis.* After screening for its secondary metabolites, a family of compounds were identified that showed correlation to antibacterial activity. The structure elucidation of these potentially new compounds, which have been isolated are still being completed.

The isolated bacteria from treated sand (dried, heated with chloramine T) and selected isolation plates (HV-agar, ISP2 and ISP4) from Isfahan desert, showed more diversity in taxonomy. The bacteria belonged to the different rare genera of *Nonomuraea*, *Herbidospora*, *Micromonospora*, *Sanguibacter*, *Agromyces*, *Gordonia*, *Prauserella*, *Dietzia*, *Jonesia* and *Micrococcus*. This study showed that with different culturing methods, we are able to isolate underexplored members of the phylum Actinobacteria that lead us to the discovery of new compounds and later antibiotics.

Two isolates of the genera *Sanguibacter* and *Agromyces* were chosen from the treated sand of Isfahan desert for polyphasic taxonomy.

4.1.2 Description of strains NS44ZA and BA

The isolate NS44ZA was discovered as closely related to the type strain *Sanguibacter keddieii* with 16S rRNA gene sequencing similarity of 98.34 %. The genus *Sanguibacter* belongs to the family of Sanguibacteraceae and encompasses five validly published species by searching for LPSN (https://www.bacterio.net/). From genotype comparison, the digital DNA-DNA hybridization value between NS44ZA and *S. keddieii* was 22.2 %, which was below the threshold of 70 %. The draft genome of NS44ZA consisted of

37 contigs with a total consensus of 3,475,186 bp and an average G+C content of 72.08 %. Two strains were different in utilising different carbon sources. Raffinose and inositol were used by *S. keddieii*, whereas NS44ZA utilised rhamnose as carbon source. *S. keddieii* had an enzyme activity for α-mannosidase and ß-glucosamidase, whereas the strain NS44ZA had no activity for those enzymes but had activity for hydrolysing gelatin and reducing nitrate. The major fatty acids in strain NS44ZA consist of *anteiso*-C15:0 (50%), *iso*-C16:0 (16%) and C16:0 (15%), whereas in *S. keddieii*, the main fatty acids are C16:0 (53%) and *anteiso*-C15:0 (11%).

The isolate BA was closely related to *Agromyces arachidis* and *Agromyces indicus* with 99.08 % and 98.80 % similarity based on complete 16S rRNA gene sequencing, respectively. Since, *A.arachidis* is not validly published, *A.indicus* was chosen for comparison in the polyphasic approach. The genus *Agromyces* belong to the family of Microbacteriaceae and encompasses thirty-six validly published species by searching for LPSN (https://www.bacterio.net/). The whole-genome of the strain BA consisted of 88 contigs with a total size of 3,870,556 bp and an average G+C content of 71.53 %. Possessing light-induced pigments, 2,4-diaminobutyric acid (DAB) in the cell wall and a fragmented vegetative mycelium are the normal characteristic of many *Agromyes* species (120), which was also observed in the strain BA. One of the phenotypic differences of the strain BA with its type strain related to the enzyme activity of α-galactosidase that was quite strong in *A.indicus* (not in BA) and ß-galactosidase, which was active in the strain BA. The major fatty acids in strain BA were *anteiso*-C15:0 (39%), *anteiso*-C17:0 (32%) and C16:0 (20%), whereas in *A.indicus*, C16:0 (38%) was the main fatty acid followed by *anteiso*-C15:0 (28%) and *anteiso*-C17:0 (22%). Other characteristics were similar and typical for *Agromyces*. Based on phenotypic and genotypic characteristics, both strains represent novel species.

4.1.3 Myxobacteria isolated from desert

In this study, thirty-two Myxobacteria have been isolated from different soil samples including desert, mangrove forest and medicinal plants soil. Four Myxobacteria have been isolated from freshwater snails. 66% of them belonged to the genus *Myxococcus.* Unfortunately, there were no interesting taxonomical Myxobacteria among the isolates. All isolates were screened in terms of production of novel secondary metabolites. One isolate 4NSX3, which showed 99.89 % similarity to *Myxococcus xanthus* (based on the complete 16S rRNA gene sequencing) showed high activity against *E.coli* TolC and

E.coli DSM 1116. However, dereplication revealed that the activity correlated to the already known compound myxovirescin C. The chances of discovering novel metabolites in Myxobacteria would increase by taxonomic distance, meaning that novel metabolites are more likely to be obtained from new genera rather than additional representatives within the same genus (21).

4.2 Isolates from mangrove forest and silver beach

Qeshm Island is the largest island in the Persian Gulf with a surface area of 1,491 square kilometers of which about 67.5 km² of it are covered by mangrove forests (Hara forests). One of the soil samples was collected from the Hara mangrove forest. Hengam island is a small island (37 km²) located in south of Qeshm island that is famous for its silver sand on the beach, which is explained by the presence of silica and aminosilicate. There was also a sand sample collected from this silver beach. Mangrove forests are unique plant community in tropical coastal areas with the most productive ecosystems (124). Some metagenomics studies about microbial communities of mangrove forests showed that Proteobacteria, Firmicutes and Actinobacteria are the dominant phyla represented in mangrove area (124). Lee *et al,*. 2014, have described mangrove environment as a rich reservoir for novel genera and species like *Mumia flava* gen. nov., sp. nov. or *Microbacterium mangrovi* sp. nov. (125). On the other hand, this study was a first trial aimed at isolating Actinobacteria from silver beach of the Hengam island. The isolated Actinobacteria from Hara mangrove forest and silver beach belonged to the genera of *Streptomyces*, *Amycolatopsis*, *Pseudonocardia* and *Actinomadura*. Two isolates of silver beach named Q1 and Q2 were chosen for polyphasic taxonomic characterization.

4.2.1 Description of strains Q1 and Q2

Two isolates Q1 and Q2 were found to be closely related to the type strains *Amycolatopsis rifamycinica* and *Pseudonocardia cypriac* with 99.03% and 99.65% similarity based on their 16S rRNA gene sequencing, respectively. Both genera belong to the family Pseudonocardiacea. *Amycolatopsis* encompasses 82 and *Pseudonocardia* 61 validly published species by searching for LPSN (https://www.bacterio. net/). Some typical phenotypic characteristics were similar in all genera such as the aminoacid in their peptidoglycan (which is *meso*-diaminopimelic acid) and galactose as one of many diagnostic whole-cell sugars. The phospholipid profile included

phosphatidylethanolamine and menaquinones mostly contained nine isoprene units (120). Genotypic analysis of dDDH showed that Q1 was more related to the type strain *Amycolatopsis saalfeldensis* DSM 44993^T with 29.0% accordance and Q2 had higher similarity to the type strain *Pseudonocardia hierapolitana* DSM 45671^T with the value of 46.1% accordance. In both cases the values were below 70% (threshold). Because of the lack of genome sequences in public databases and low-quality of available genome sequences, obtaining the complete genomes for taxonomic purposes without the results of phenotypic and chemotaxonomic analysis would be impractical (34). Both isolates grew well on the plates with pH of 8,9 and 10, which was predictable, because the silver beach sand also had a pH of 8.49 without ion exchanger. Based on Bergey's systematic bacteriology, the genus *Amycolatopsis* can produce aerial mycelium and the substrate mycelium fragments into irregular rods. In the genus *Pseudonocardia* vegetative mycelium can fragment and there is no spore chain produced on aerial mycelium (120). Both strains Q1 and Q2 produced no aerial mycelium and their substrate mycelium fragmented in rods under the light microscope. For chemotaxonomic analysis, the isolate Q1 was compared to six type strains: *A. rifamycinica* DSM 46095, *A. rhabdoformis* SB026, *A. kentuckyensis* DSM 44652, *A. lexingtonensis* DSM 44653, *A. pretoriensis* DSM 44654 and *A. balhimycina* DSM 44591. All type strains were cultured under the same culture conditions. Based on enzyme activities, the strain Q1 showed more similarities to type strains *A. rifamycinica* and *A. rhabdoformis*, for example, possessing enzymes like α-fucosidase. Strain Q1 showed difference with all type strains for its β-glucoronidase activity. The predominant menaquinone and polar lipids were close to other type strains but the fatty acid pattern had more differences that were similar to *A. rifamycinica.* The isolate Q2 was compared in all analyses with four type strains: *P. cypriaca* DSM 45511, *P. hierapolitana* DSM 45671, *P. zijingensis* DSM 44774 and *P. adelaidensis* DSM 45352. For enzyme activity, it showed more similarity to the type strains of *P. hierapolitana* and *P. adelaidensis* especially for nitrate reduction and positive activity of pyrazinamidase. The strain Q2 was able to produce β-glucoronidase and β-glucosidase, which were not produced in other type strains. The most similar fatty acid pattern was observed in Q2 and *P. cypriaca.*

From 2008 until 2018, rare actinomycetes including the genera *Micromonospora*, *Actinomadura* and *Amycolatopsis* attracted the attention of many researchers due to their potential in producing bioactive compounds (126). The genus *Amycolatopsis*

specifically is an interesting genus for the pharmaceutical industry. There are many important antibiotics produced by the members of this genus, such as rifamycin (127), vancomycin (128), balhimycin (129) and dethymicin (130,131). Strain Q1 showed antibacterial activity against Gram-positive bacteria and could be potentially a good candidate for further investigation on its secondary metabolites.

Pseudonocardia is another interesting genus of rare Actinobacteria, which is known to produce novel bioactive compounds such as pseudonocardide A–G (132), branimycin B-C (133), pseudonocardian A–C (134). Strain Q2 showed activity against Gram-positive bacteria and need further investigation for its bioactive compounds.

4.3 Isolates from the soil of medicinal plants

Iran has a rich flora and strong background in consuming medicinal plants for therapeutic usage (135). There are some local plants in non-popular cities of Iran where locals would know about their benefits. In order to screen associated Actinobaceria of these local plants, the soil samples from their root, their leaves and some wooden part of their stem were collected. The samples were collected from Almond, Mord (kind of myrtle), Khesht, Oak, Astragalus and Safran. The most abundant genera isolated from them were *Streptomyces* (16 different species), *Lentzea* (2 species) and *Amycolatopsis* (1 specie). Although in culture-dependent studies, the most of identified isolates belonged to the genus *Streptomyces*, but culture-independent studies using 16S rRNA gene amplicon sequencing, have shown that this genus formed a small portion of actinobacterial communities in different habitats like soil, plant or marine sediments (136). The genus *Streptomyces* provides half of all known actinobacterial compounds (137). Although, from 1985 there was a decline in discovery of new antibiotics from them but they continue to promising until date. Recently a new strain of *Streptomyces sioyaensis*, named *Streptomyces* sp. TM32 was isolated from the rhizosphere of Curcuma longa L., a medicinal plant, which was also used in cooking and has strong antimicrobial activities against both human and plant pathogens (138). Among all of the isolates from the medicinal plants collected from cities Yasooj and Tafresh, one isolate from Astragalus plant showed strong antifungal activity and low phylogenetic similarity (based on 16S rRNA gene sequencing). Therefore, it was chosen for further studies.

4.3.1 Strain NS40 and its secondary metabolites

This strain NS40 showed the highest similarity to *Streptomyces badius* DSM 40139 by 98.55% based on 16S rRNA gene sequencing. The result of DDH showed the value of 25% between these two strains, which was below the threshold of 70%. Two other type strains were chosen for the polyphasic taxonomic approach (*Streptomyces sindenensis* and *Streptomyces parvus*). The phylogenetic results revealed that strain NS40 belonged to another clade that was not close enough to other three type strains, which were suggested by the EzTaxon server. The phenotypic analyses were in line with genotypic analyses and showed different enzyme activity and fatty acid patterns for the strain NS40. High-performance liquid chromatography (HPLC) and high-resolution electrospray ionization-mass spectrometry (HR-ESI-MS) analyses of crude extract fractions resulted in the detection of an already known compound, Amphotericin A. This antifungal compound was isolated from *Streptomyces nodosus* was collected from soil samples in Orinoco River region of Venezuela (114). Previously the anti-candidal activity of *Astragalus verus* was studied in guinea pig model for systematic candidiasis (139), but to the best of our knowledge there has been no study aimed to culture Astragalus-associated Actinobacteria.

4.4 Isolates from freshwater snails

Actinobacteria as a defensive symbiont has captured the attention of researchers for several decades. This interaction has been well-studied in some invertebrates including insects within Actinobacteria that protect the host or the food source by producing antibiotics (31). After insects, Mollusca is the second-largest phylum of the invertebrates and there exist only few studies on the most diverse group of them, gastropoda. Cone snails in marine habitats have been studied so far for the associated Actinobacteria and their secondary metabolites (140). The neuroactive thiazoline metabolites pulicatins A-E have been extracted from a novel *Streptomyces* sp. isolated from Cone snails (141). In this study, the diversity and antimicrobial potential of freshwater snails-associated Actinobacteria were assessed by culture-dependent and culture-independent approaches, respectively.

4.4.1 Culture-dependent analyses

Totally 28 Actinobacteria (including genera *Streptomyces*, *Micromonospora*, *Nocardia*, *Kitasataspora* and *Nonomuraea*) and 4 Myxobacteria (genus *Myxococcus*)

were isolated from different genera of freshwater snails that were collected from different regions of Iran and Germany. Among all of them, one isolate 7NS3 showed good antibacterial activity and low similarity 99.2% to *Streptomyces seoulensis* NRRL B-24310^T. Therefore, it was chosen for polyphasic taxonomy and further investigation.

4.4.2 Description of strain 7NS3 and its secondary metabolites

Strain 7NS3 was isolated from the freshwater snail *Physa acuta* collected in Remlingen-Semmenstedt, Germany. *Physa acuta* has a widespread distribution across the world due to its rapid generation time and high productivity, which increase their population, it additionally acts as an intermediate host of some trematode diseases, such as echinostomiasis and fascioliasis (75). Although some other strains were isolated from this snail, the highest activity was observed for the crude extract of strain 7NS3, with an MIC value of 8.3 µg/ml and 0.52 µg/ml against *S. aureus* (Newman) and *B. subtilis* DSM 10^T, respectively. The whole-genome sequence of isolate 7NS3 contained a total genome size of 6.85 Mbp with a GC content of 71.6% and the dDDH result of *Streptomyces* sp. 7NS3 and *S. seoulensis* NRRL B-24310^T showed the value of 29.5%, which was below the threshold of 70%. The results of high-performance liquid chromatography (HPLC) and high-resolution electrospray ionization-mass spectrometry (HR-ESI-MS) analyses of crude extract fractions resulted in the detection of four compounds, one of which matched the compound characteristics of the emycin A, an angucycline-like aromatic polyketide. Genome mining studies based on the whole-genome sequence of 7NS3 resulted in the identification of a gene cluster potentially coding for emycin A biosynthesis. In our recently published work, we have described more details about this strain (142).

4.4.3 Culture-independent analyses

To obtain the composition of the bacterial community, especially of the phylum Actinobacteria, a metagenome approach was performed (142). The amplicon reads of the V3 region of the 16S rRNA gene grouped 21 phyla with the most abundant phyla Proteobacteria (65%), Bacteroidetes (17%), Cyanobacteria and Chloroplast (8%) and Firmicutes (6%). The phylum Actinobacteria encompasses just 2% of the bacterial population with the predominant family of Kineosporiaceae (26%), Microbacteriaceae (20%), Acidimicrobiaceae (18%) and Nocardioidaceae (10%). The family Streptomycetaceae represented just four identified sequences. Previously, there were some studies on microbial communities of freshwater snails, for example in *Radix*

auricularia, the predominant phyla were Proteobacteria (35%), Cyanobacteria (16%), Firmicutes (14%), Chloroflexi (9%) and Actinobacteria (8%) (143). Investigation into the intestinal bacterial community in planorbid snails revealed that Gammaproteobacteria, Bacteroidetes and Acidobacteria were the predominant phyla (144). Host diet, physical and chemical conditions of the intestine, age and many other factors could affect the indigenous gut microbial community (144,145). The other studies about microbiome of freshwater snails were mostly limited to exploring cellulolytic bacteria or determining the roles of gut bacteria in physiology and immunology of the host. Therefore, it is suggested not only to rely on culture-independent studies but also to combine it with culture-dependent studies to make the best use of the isolates for biotechnology usage especially in drug discovery.

5 Conclusion

The aim of this work was to isolate new strains of two promising groups of bacteria in terms of producing novel bioactive compunds. Myxobacteria and Actinobacteria are two groups that we have focused on. The samples were collected from uncommon habitats of Iran. Some non-typical environments like the desert, mangrove forest, medicinal plants and the beach sand. On the other hand, investigating about freshwater snails was captured our attention. The bacterial diversity of freshwater snails was reported before but studies on culture-dependent approaches were limited.

In the context of this work, no interesting Myxobacteria was isolated. However, there were quite interesting species of Actinobacteria that belonged to non-*Streptomyces* group. Among them 5 novel species were identified and characterized with whole-genome sequencing and polyphasic taxonomy approach. The strains 4NS15, Q1, Q2, 44NSZA, BA belonged to the genera *Kibdelosporangium*, *Amycolatopsis*, *Pseudonocardia*, *Sanguibacter* and *Agromyces* respectively. Besides, their interesting taxonomical features, their ability to produce active secondary metabolites were tested. The crude extract of strain 4NS15 showed strong bioactivity against Gram-positive bacteria and after fermentation of 20 Liter of its crude extract, a family of compounds belonged to the class of terpenoids were isolated and purified. The structure elucidation of this family is still undergoing.

The other project on freshwater snail *Physa acuta* resulted in identifying a new species of the genus *Streptomyces* that its crude extract showed good activity against Gram-positive bacteria and dereplication showed the activity came from an already known compound named Emycin A that can be isolated and purified in future.

To conclude, it can be confirmed that the search for new natural substances in unexplored habitats is proving successful and different media and culture condition are needed to target taxonomical interesting strains. Furthermore, freshwater snails should consider as a promising reservoir for isolating new strains that may have new bioactive compounds.

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7 Appendix

Table S1 List of all used media

Isolation and purification media for actinobacteria	
GYM Medium	Maintenance and reactivation
Glucose	4.0 g/l
Yeast extract	4.0 g/l
Malt extract	10.0 g/l
$CaCO_3$	2.0 g/l
Agar	12 g/l
Deionized Water	1000 ml
5336 + cyclo	Actinobacteria isolation medium
Soluble starch	10.0 g/l
Casein (Pepton Typ M)	1.0 g/l
K_2HPO_4	0.5 g/l
$MgSO_4$ x 7 H_2O	5.0 g/l
Agar	20.0 g/l
Deionized water	1000
Cyclohexamide solution (50 mg/ml in methanol) added after autoclave	2ml

Non-Streptomyces isolation medium	
Humic Acid-Vitamin Agar (HV)	
Humic acid	10g(dissolved in 10ml of 0.2N NaOH)
Na_2HPO_4	0.5g
KCI	1.71g
$MgSO_4.7H_2O$	0.05g
$FeSO_4.7H_2O$	0.01g
$CaCO_3$	0.02g
Cyelohexinaide	50 mg
Agar	18g
Distilled water	1L
B-vitamins	0.5mg each of thiamine-HCl, riboflavin, niacin, pyridoxin-HC1, inositol, Ca-pantothenate, p- aminobenzoic acid, and 0.25mg of biotin
pH	7.2
ISP medium (International *Streptomyces* Project)	
ISP2/ Yeast malt agar	Maintenance and taxonomy
Malt extract	10.0 g/l
Yeast extract	4.0 g/l
Glucose	4.0 g/l
Agar	15.0 g/l

Deionized Water pH	1000 ml 7
ISP3/ Oat meal agar Oatmeal (Quaker white oats) Agar Deionized Water Trace salt solution pH	Maintenance and taxonomy 20.0 g/l 18.0 g/l 1000 ml 1 ml 7.2
(Trace salt solution ISP3) $FeSO_4$ x $7H_2O$ $MnCl_2$ x $4H_2O$ $ZnSO_4$ x $7H_2O$ Deionized water	 0.1 g 0.1 g 0.1 g 100ml
ISP 4 Soluble starch $(NH_4)2SO_4$ K_2HPO_4 $MgSO_4$ x $7H_2O$ NaCl $CaCO_3$ Agar Deionized Water pH	Maintenance and taxonomy 10.0 g/l 2.0 g/l 1.0 g/l 1.0 g/l 1.0 g/l 2.0 g/l 20.0 g/l 1000 ml 7.3
ISP5 L-Asparagine Glycerol K_2HPO_4 Trace salt solution Agar Deionized Water pH	Maintenance and taxonomy 1.0 g/l 10.0 g/l 1 g/l 1ml/l 20 g/l 1000 ml 7.2
(Trace salt solution ISP5) $FeSO_4$ x 7 H_2O $MnCl_2$ x 4 H_2O $ZnSO_4$ x 7 H_2O Deionized water	 1.0 g 1.0 g 1.0 g 100ml
ISP6 / Peptone Iron Agar Peptone Proteose Peptone Ferric ammonium citrate Sodium glycerophosphate Sodium thiosulfate-5-hydrate Yeast extract Agar	Production of melanoid pigment 15.0 g/l 5.0 g/l 0.5 g/l 1.0 g/l 0.126 g/l 1.0 g/l 20 g/l

Deionized Water pH	1000 ml 7.2
ISP7/ Oat meal agar Glycerol L-Tyrosine L-Asparagine K_2HPO_4 NaCl $FeSO_4$ x 7 H_2O Trace Salt solution (5343) Agar Deionized water pH	Production of melanoid pigment 15 g/l 0.5 g/l 1.0 g/l 0.5 g/l 0.5 g/l 0.01 g/l 1.0 ml/l 20.0 g/l 1000 ml 7.3
5343 FeSO4 x 7 H2O $ZnSO_4$ x 7 H2O $MnCl_2$ x 4 H2O Deionized water	Trace element solution 4 1 g/l 1 g/l 1 g/l 1000 ml
Synthetically Suter Medium (SSM+T) Glycerol Tyrosine L-arginine L-glutamic acid L-methionine L-isoleucine K_2HPO_4 $MgSO_4$ x 7 H_2O Trace element solution 2 (5341) Agar -Control medium is prepared without tyrosine (SSM-T)	Production of melanoid pigment 15.0 g/l 1.0 g/l 5.0 g/l 5.0 g/l 0.3 g/l 0.3 g/l 0.5 g/l 0.2 g/l 1.0 ml/l 20 g/l
5341 $CuSO_4$ x 5 H_2O $CaCl_2$ x 2 H_2O FeSO4 x 7 H_2O $ZnSO_4$ x 7 H_2O $MnSO_4$ x 7 H_2O Deionized water	Trace element solution 2 10.0 g/l 10 g/l 10 g/l 10 g/l 40 g/l 1000 ml

Production media	
Medium 5254 Glucose Soymeal	(Metabolite production) 15.0 g/l 15.0 g/l

Corn steep liquor	5.0 g/l
$CaCO_3$	2.0 g/l
NaCl	5.0 g/l
Deionized water	1000 ml
pH	7.0
Medium 5294	(Metabolite production)
Starch (soluble)	10.0 g/l
Yeast extract	2 g/l
Glucose	10 g/l
Glycerol	10 g/l
Corn steep liquor	2.5 g/l
Peptone (Marcor S)	2.0 g/l
NaCl	1 g/l
$CaCO_3$	3.0 g/l
Deionized water	1000 ml
pH	7.2
Medium 5333	(Metabolite production)
Starch (soluble)	15 g/l
Yeast extract	4 g/l
K_2HPO_4	1 g/l
$MgSO_4$ x 7 H_2O	0.5 g/l
Deionized water	1000 ml
Ph	7.0
Soymeal (Mannit medium)	(Metabolite production)
Soymeal	20 g/l
Mannit	20 g/l
Glucose	4 g/l
Deionized water	1000 ml

Isolation and purification media for myxobacteria	
Water-agar	(predators)
$CaCl_2$ x $2H_2O$	0.15%
$MgSO_4$ x $7H_2O$	0.15%
Hepes	50 mM
vitamin solution (Schlegel)	1ml/l
Agar	1.8%
pH	7.2
Vitamin solution (Schlegel)	
Biotin	0.2%
Nicotinsäure	2.0%
Thiamin	1.0%
4-Aminobenzoesäure	1.0%
Pantothenat	0.5%
Pyridoxamin	5.0%
Cyanocobalamin	2.0%

The vitamin solution is sterilized by filtration and stored at 4°C	
ST21 -agar Solution A: K_2HPO_4 yeast extract (Difco) or 1% baker´s yeast (see VY/2 medium) Agar Solution B: KNO_3 $MgSO_4 \cdot 7H_2O$ $CaCl_2 \cdot 2H_2O$ $FeCl_3$ $MnSO_4 \cdot 7H_2O$ Combine solutions A and B and add 1 ml of trace element solution per liter of medium.	Cellulose decomposers 0.1% 0.002% 1.0% 0.1% 0.1% 0.1% 0.02% 0.01%
VY/2-agar baker's yeast $CaCl_2$ x $2H_2O$ (vitamin B12) Hepes Agar (Difco) Sterilize vitamin B12 separately by filtration. Prepare and store yeast cells as autoclaved stock suspension (50 g baker's yeast/100 ml distilled water. adjust pH to 6.5 and autoclave). Adjust pH of medium to 7.2 with KOH before. and after autoclaving and cooling to 50°C (use pH-indicator paper).	 10 ml/l 0.1% 0.5mg/l 50mM 1.6%

Metabolic production media for myxobacteria	
Myxovirescin - Medium Casein peptone $CaCl_2$ x $2H_2O$ $MgSO_4$ x $4H_2O$ $CoCl_2$ Hepes pH	 1.0% 0.005% 0.025% 1mg/l 100mM 7.0
P – Medium Peptone (Marcor M) Starch (Cerestar) Probion Yeast extract (Marcor typ 9000) $CaCl_2$ x $2H_2O$ MgSO4 x $4H_2O$	 0.2% 0.8% 0.4% 0.2% 0.1% 0.1%

HEPES Fe-EDTA pH	100mM 8mg/l 7.5
H-Medium Soy flour (degreased) Glucose monohydrat Starch (Cerestar) Yeast extract (Marcor typ 9000) $CaCl_2$ x $2H_2O$ $MgSO_4$ x $4H_2O$ HEPES Fe-EDTA pH	 0.2% 0.2% 0.8% 0.2% 0.1% 0.1% 50mM 8mg/l 7.4
POL - Medium Probion Starch (Cerestar) $CaCl_2$ x $2H_2O$ $MgSO_4$ x $4H_2O$ HEPES Ph	 0.3% 0.3% 0.05% 0.2% 50mM 7.2

Table S-2 List of 80 isolated Actinobacteria

City	Code	Type of sample	Isolated actinobacteria	Similarity 16S rRNA (Partial)
Yasooj	NS1	Plant leaf (Mord)	*Streptomyces galilaeus*	100
	NS3	Almond tree soil		100
	NS49	Plant leaf (Mord)		100
	NS65	Plant leaf (Mord)		99.5
	NS57	Almond tree soil		100
	NS2	Plant leaf (Khesht)	*Streptomyces exfoliatus*	100
	NS8	Plant leaf (Mord)		100
	NS18	Plant leaf (Mord)		99.79
	NS24	Plant leaf (Mord)		99.76
	NS4	Almond tree soil	*Streptomyces griseus*	99.89
	NS6	Plant leaf (Khesht)		99.88
	NS22	Plant leaf (Khesht)		99.88
	NS47	Plant leaf (Mord)		99.88
	NS51	Almond tree soil		99.88
	NS55	Almond tree soil		100
	NS13	Almond tree soil	*Streptomyces youssoufiensis*	99.78
	NS28	Almond tree soil		99.78
	NS16	Almond tree soil	*Streptomyces albidochromogenes*	99.89

	NS20	Plant leaf (Mord)	*Streptomyces finlayi*	100
	NS23	Oak tree soil	*Streptomyces anulatus*	99.52
	NS35	Plant leaf (Khesht)		99.52
	NS5	Lichen		99.22
	NS48	Almond tree soil	*Streptomyces alboniger*	99.32
	NS31	Plant leaf (Mord)	*Streptomyces plumbiresistens*	100
	NS33	Almond tree soil	*Streptomyces violarus*	99.76
	NS40	Astragalus soil	*Streptomyces badius*	98.55
	NS60	Lichen	*Streptomyces cinereospinus*	99.73
	NS63	Lichen		99.62
	NS27	Plant leaf (Mord)	*Streptomyces turgidiscabies*	99.1
	NS61	Lichen	*Streptomyces pratensis*	99.65
	NS36	Almond tree soil	*Lentzea violacea*	98.9
	NS54	Almond tree soil	*Lentzea californiensis*	100
	NS66	Plant leaf (Mord)	*Amycolatopsis albidoflavus*	99.79
Tafresh	NS9	Safran soil	*Streptomyces galilaeus*	100
	NS17	Safran soil	*Streptomyces roseolus*	99.58
Lorestan	6NS1	Forest soil	*Streptomyces griseus*	99.88
	6NS6	Forest soil	*Streptomyces zaomyceticus*	100
	6NS4	Forest soil	*Nocardiopsis symnematafamus*	99.54
Boushehr	NS10	Sand	*Streptomyces griseus*	99.88
	NS21	Soil		99.88
	NS12	Sand	*Streptomyces tanashiensis*	99.88
	NS29	Soil		99.88
	NS14	Soil	*Streptomyces fragilis*	99.52
	NS32	Sand	*Streptomyces peucetius*	99.84
	NS41	Sand	*Streptomyces coelicoflavus*	100
	NS46	Soil	*Streptomyces pratensis*	99.73
	NS37	Sand	*Nocardiopsis umidischolae*	99.39
	NS30	Soil	*Nocardiopsis dassonvillei*	100
Tehran	NS42	Cocroach	*Streptomyces albidoflavus*	99.33
	NS43	Cocroach		99.38
	NS58	Cocroach		99.51
Saravan	6NS8	Soil	*Streptomyces olivaceus*	100
	6NS10	Soil	*Streptomyces inddigenes*	100
Qeshm	NS7	Sand	*Streptomyces cinereospinus*	99.54
	NS11	Soil		99.54
	NS53	Mangrove forest		99.63
	NS39	Mangrove forest	*Streptomyces indiaensis*	99.1
	NS25	Sand from silver island	*Actinomadura apis*	99.23

	Q1	Sand from silver island	*Amycolatopsis rifamycinia*	98.67
	Q2	Sand from silver island	*pseudonocardia cypriaca*	99.39
Semnan	NS62	Desert soil	*Streptomyces griseoflavus*	99.88
	NS64	Desert soil		99.87
	NS34	Desert soil	*Kribbella pittospori*	99.88
	NS59	Desert soil	*Streptomyces specialis*	98.03
Isfahan	NS38	Desert soil	*Nonomuraea harbinensis*	99.62
	NS44	Desert soil	*Herbidospora sakaeratensis*	97.64
	NS45	Desert soil	*Micromonospora echinofusca*	99.63
	44ZA	Desert soil	*Sanguibacter keddieii*	98.34
	BA	Desert soil	*Agromyces arachidis*	99.58
	441B	Desert soil	*Goordonia alkanivomans*	100
	sef	Desert soil	*Prauserella alba*	100
	SO	Desert soil	*Dietzia maris*	99.7
	44MX	Desert soil	*Jonesia quighaiensis*	100
	44Z	Desert soil	*Micrococus aloverae*	100
Kerman	4NS15	Desert soil	*Kibdelosporangium aridum*	98.9
	4NS12	Desert soil	*Saccharothrix texasensis*	99.87
Braunschweig	3B1	Soil	*Streptomyces dijakartensis*	99.49
	F1Y	Soil	*Isopetricola dokdonensis*	99.79
	F1OB	Soil	*Glutamicibacter habphtocola*	99.79

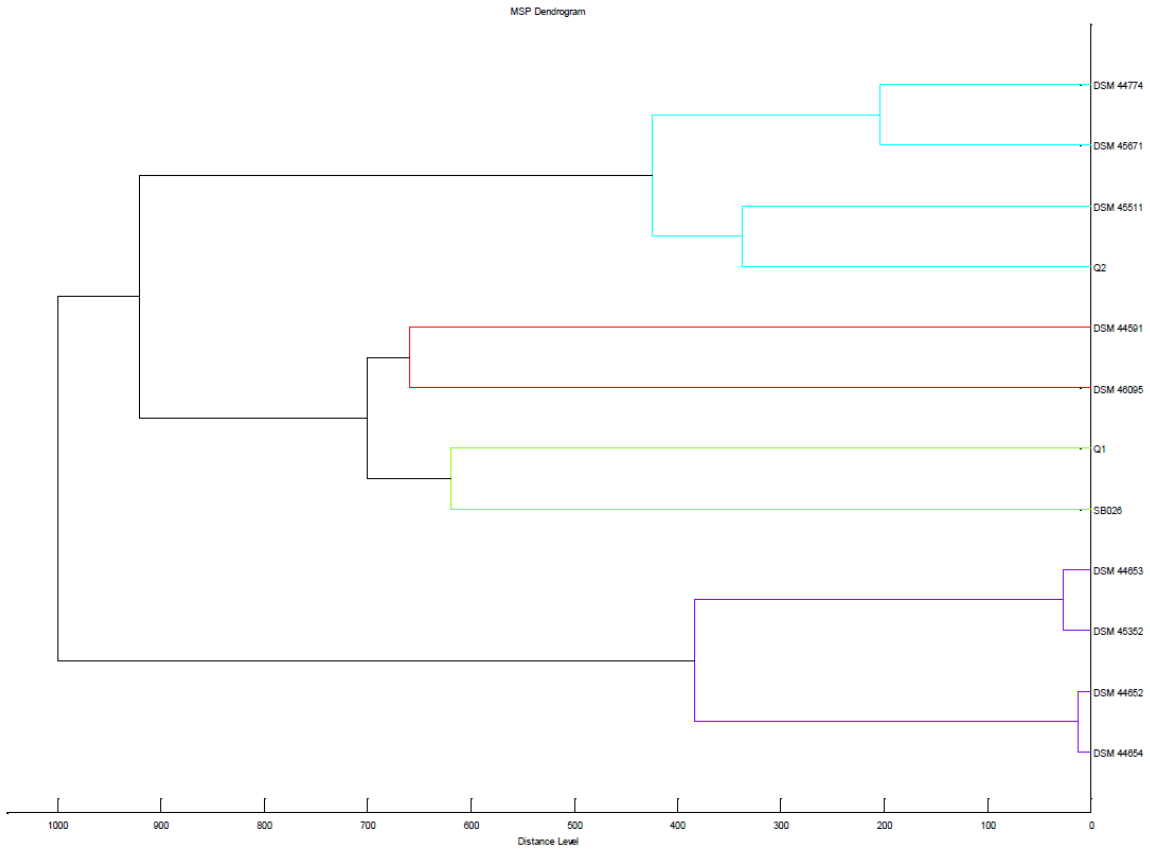


Figure S1 Dendrogram obtained by cluster analysis of MALDI-TOF MS spectra of Q1 and Q2. Five type strains of each species were analyzed. Distance is displayed in relative units.

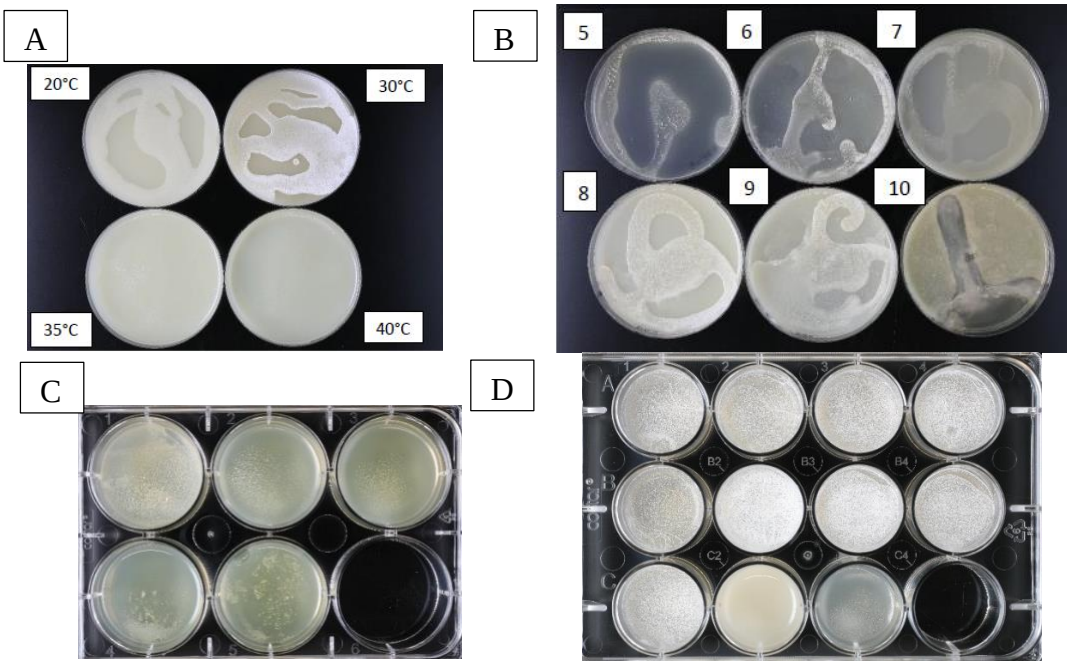


Figure S2 Results of A) temperature, B) pH, C) salt and D) carbon utilisation of Q1

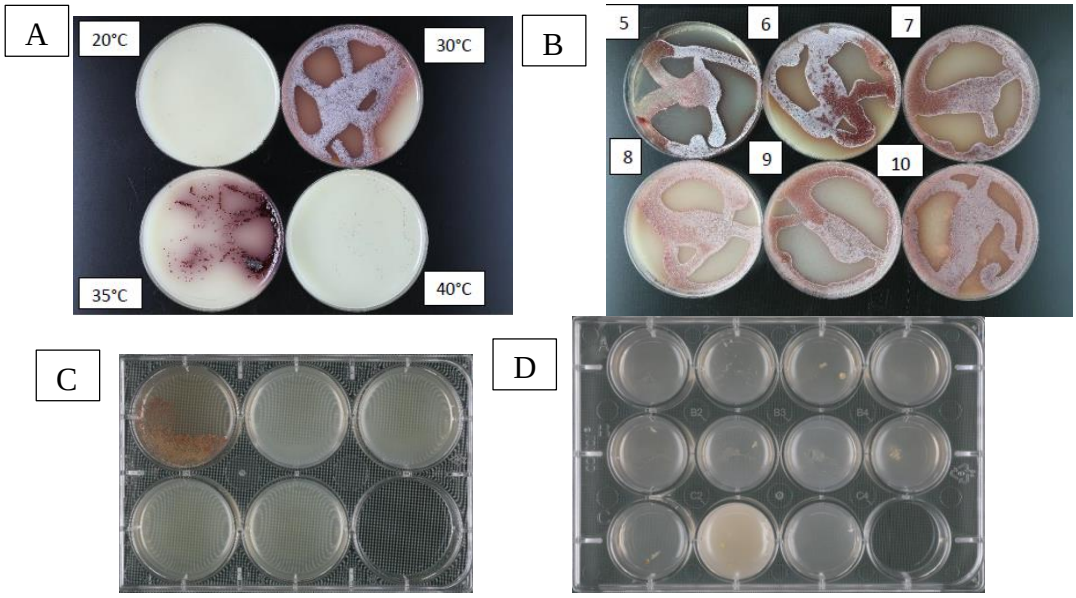


Figure S3 Results of A) temperature, B) pH, C) salt and D) carbon utilisation of Q2

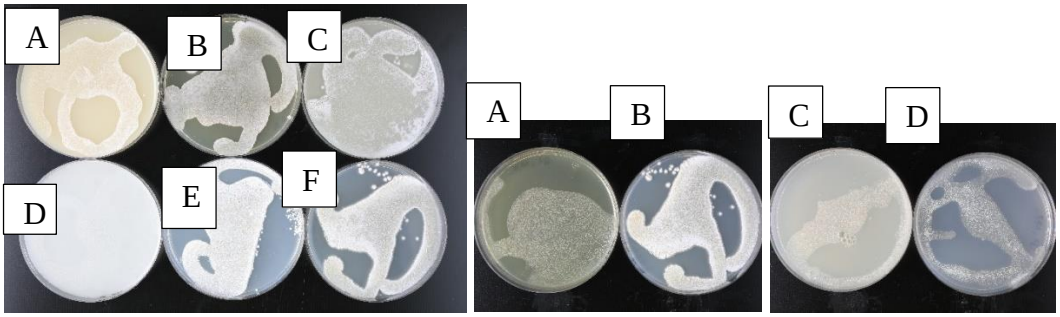


Figure S4 ISP plates of Q1 (left) A)GYM, B)ISP2, C)ISP3, D)ISP4, E)ISP5, F)ISP7 (right) A)ISP6, B)ISP7, C)SSM+, D) SSM-

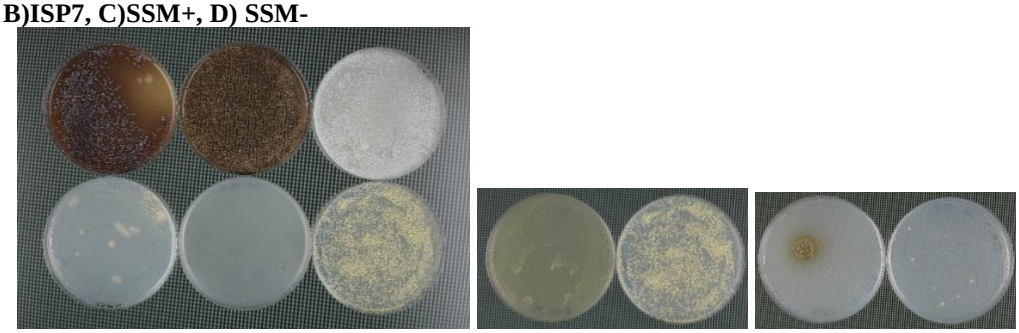


Figure S5 ISP plates of Q1 (left) A)GYM, B)ISP2, C)ISP3, D)ISP4, E)ISP5, F)ISP7 (right) A)ISP6, B)ISP7, C)SSM+, D)SSM-

Figure S6 Enzyme activity of Q1 and related type strains

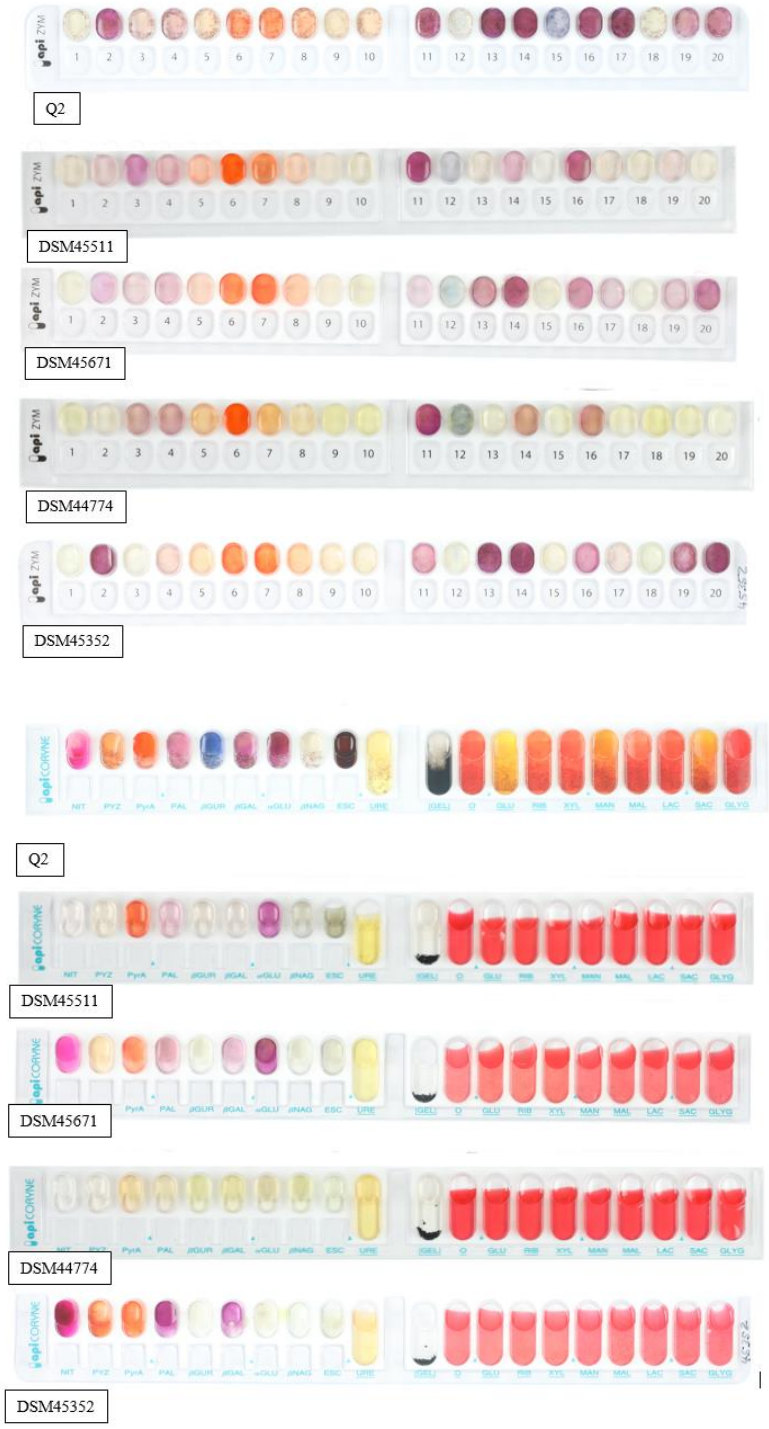


Figure S7 Enzyme activity of Q2 and related type strains

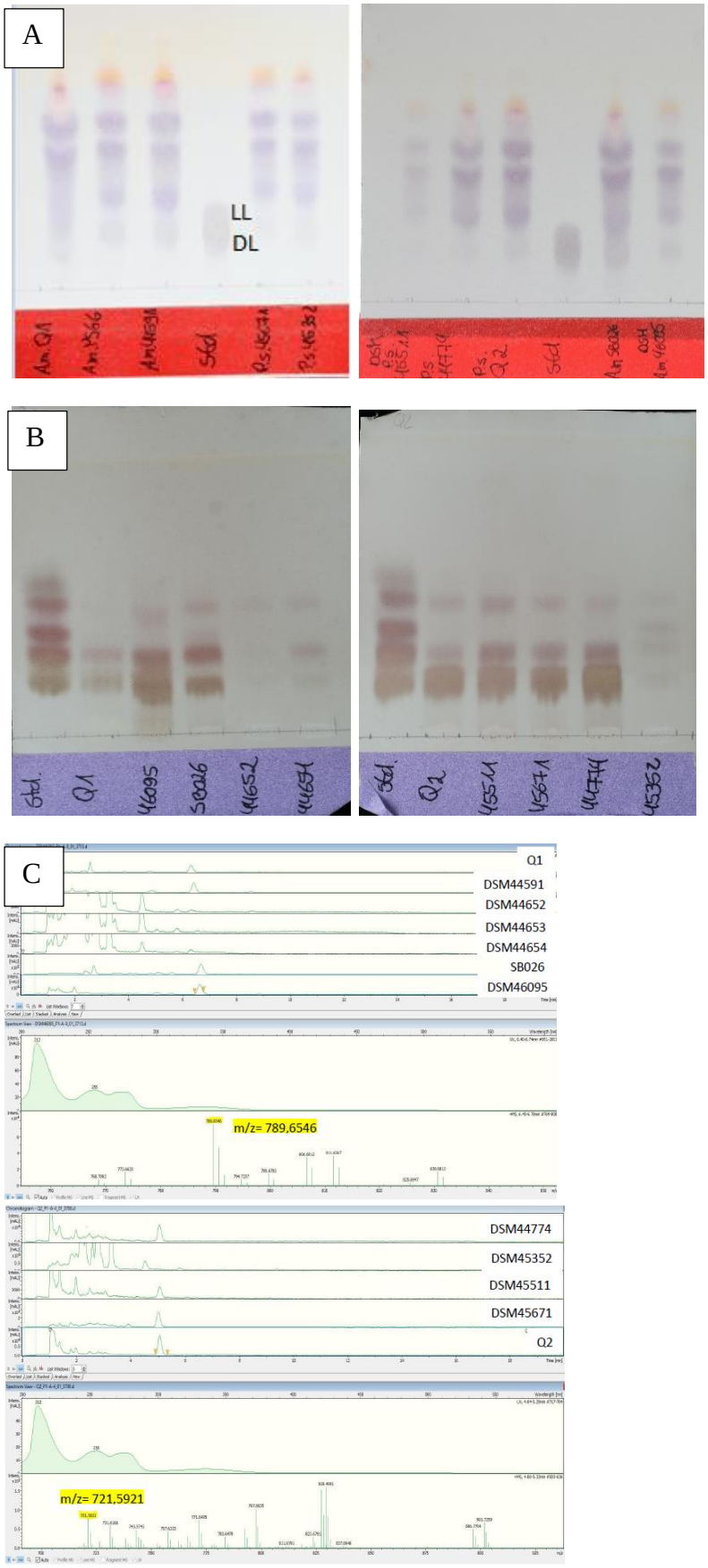


Figure S8 A) aminoacid, B) sugar and C) menaquinone results for strain Q1 (left) and Q2 (right)

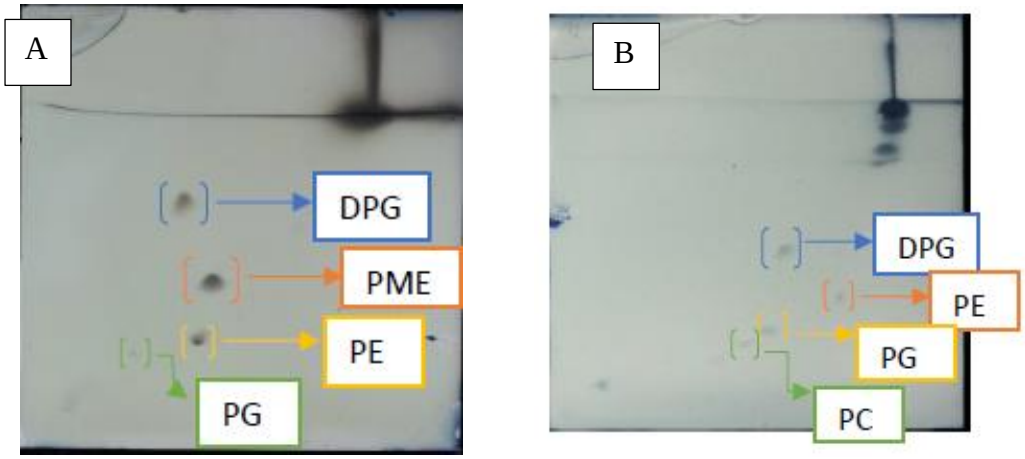


Figure S9 Identified polar lipids in Q1 (A) and Q2 (B)

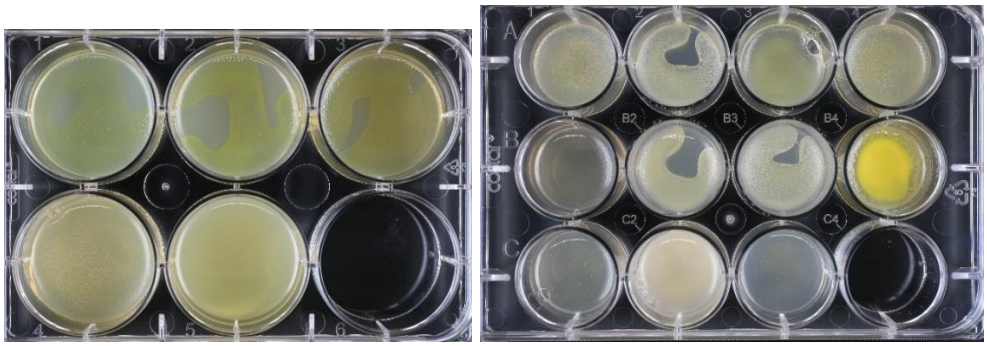


Figure S10 Results of salt and carbon utilisation of strain NS44ZA

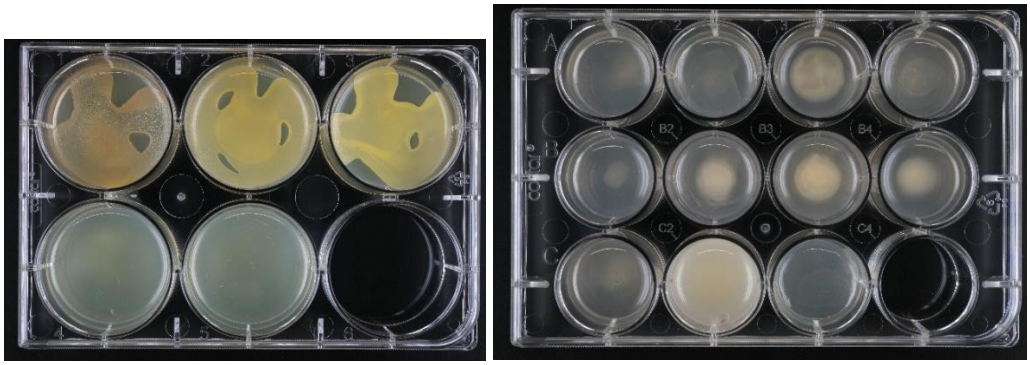


Figure S11 Results of salt and carbon utilisation of strain BA

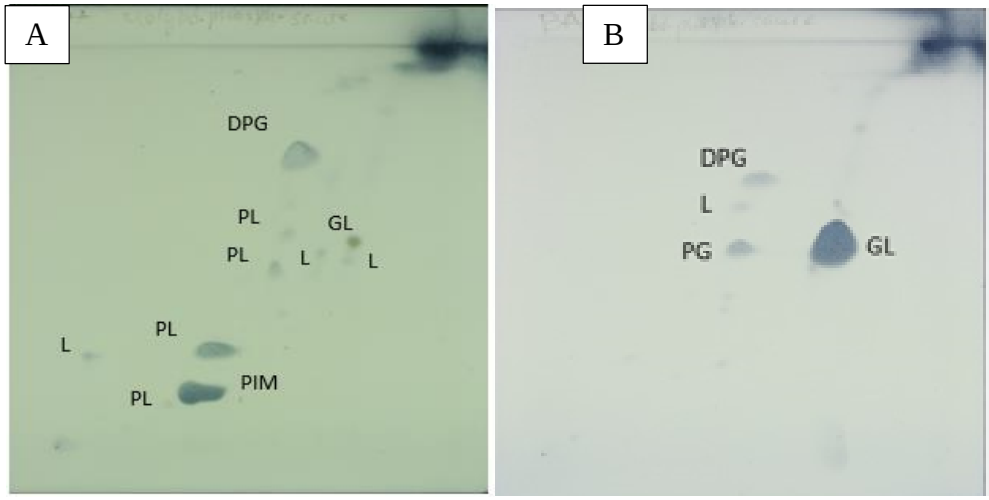


Figure S12 Identified polar lipids in strain A) NS44ZA and B) BA

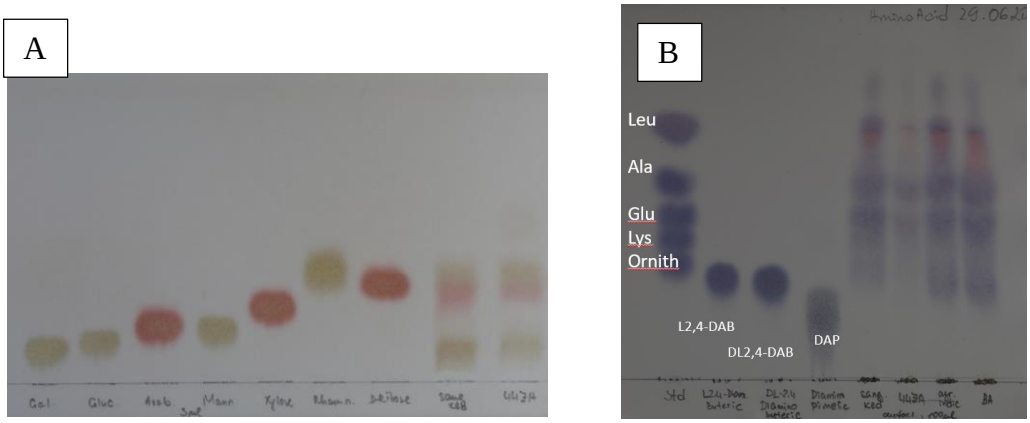


Figure S13 Identified A) Sugars and B) Aminoacids in strains NS44ZA and BA

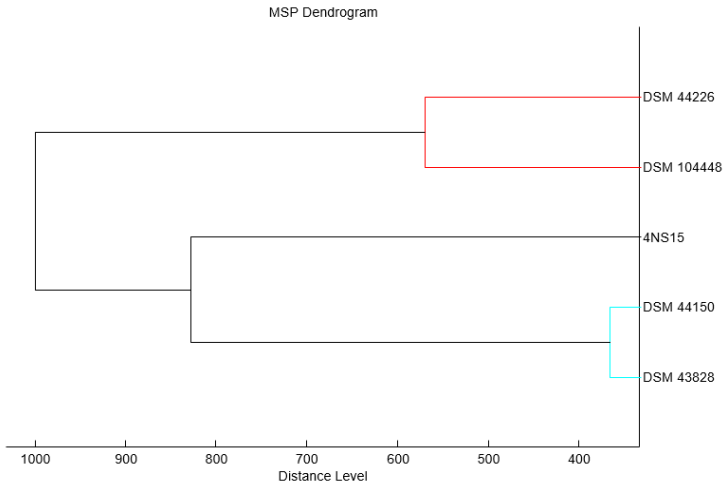


Figure S14 Dendrogram obtained by cluster analysis of MALDI-TOF MS spectra of 4NS15

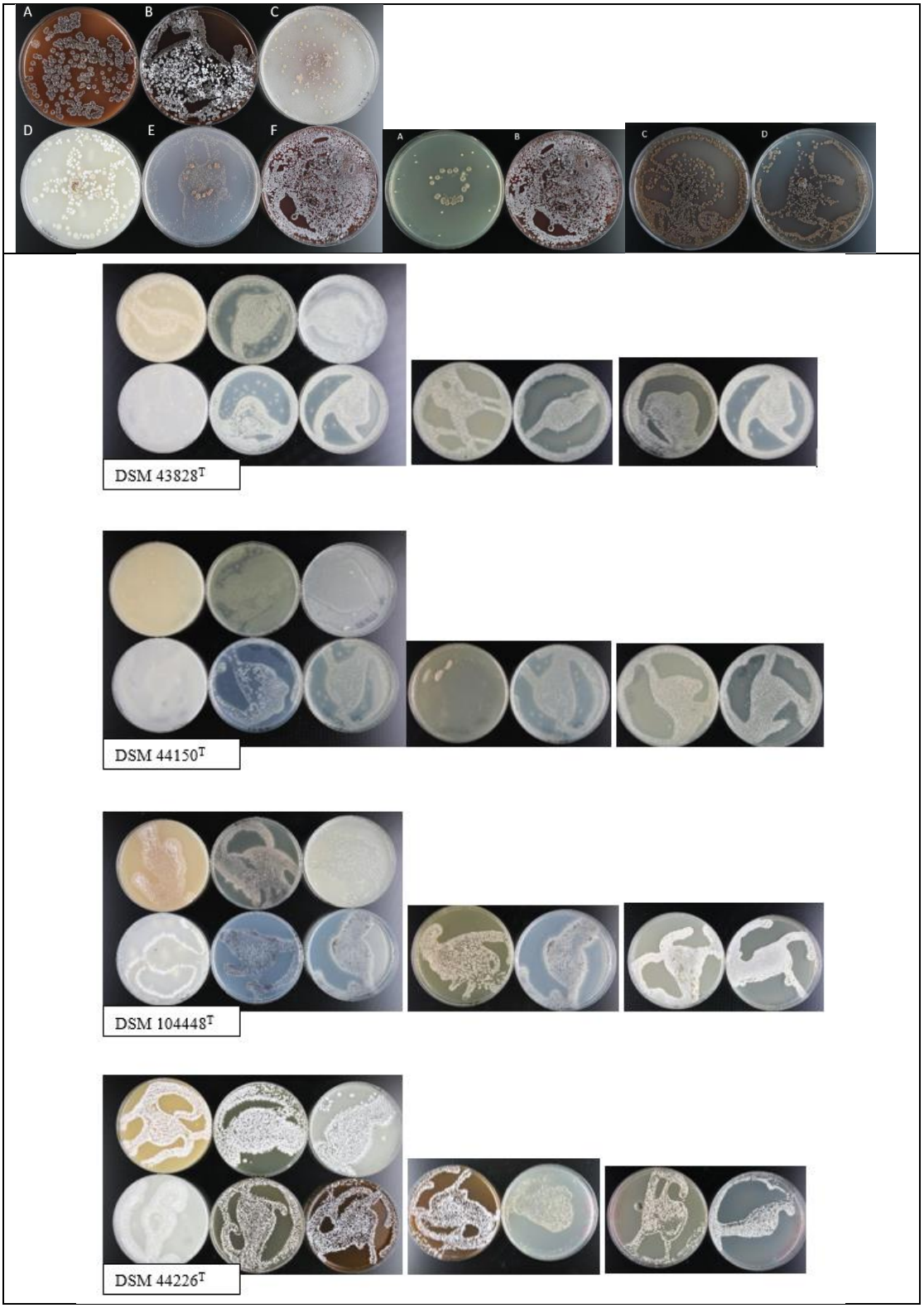


Figure S15 ISP plates of 4NS15 and type strains (left) A)GYM, B)ISP2, C)ISP3, D)ISP4, E)ISP5, F)ISP7 (right) A)ISP6, B)ISP7, C)SSM+T, D)SSM-T

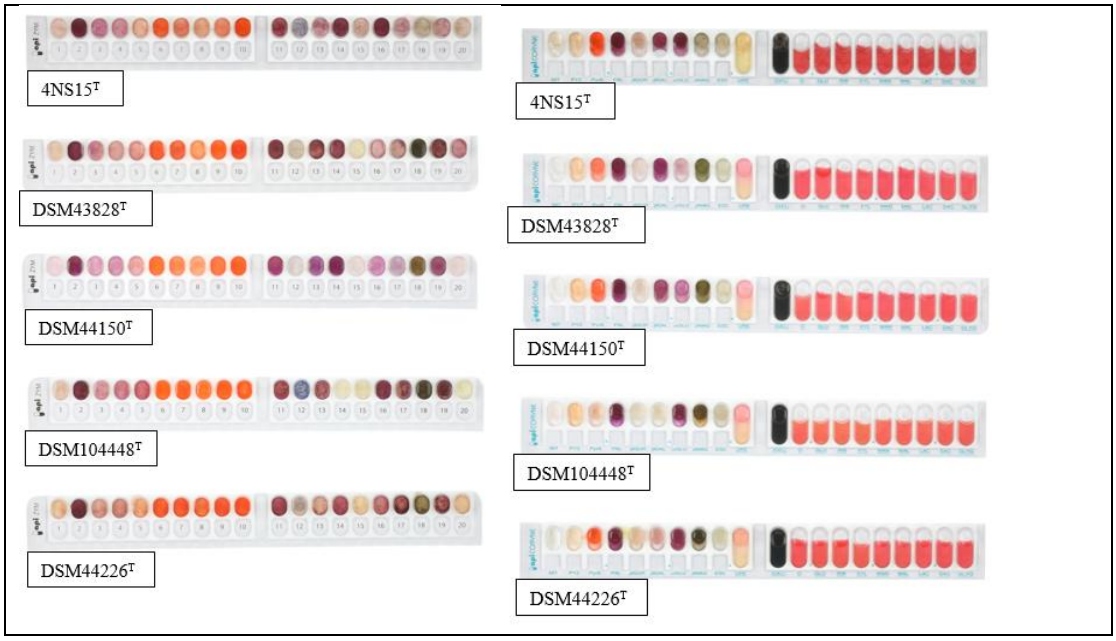


Figure S16 Enzyme activity of strain 4NS15 and related type strains

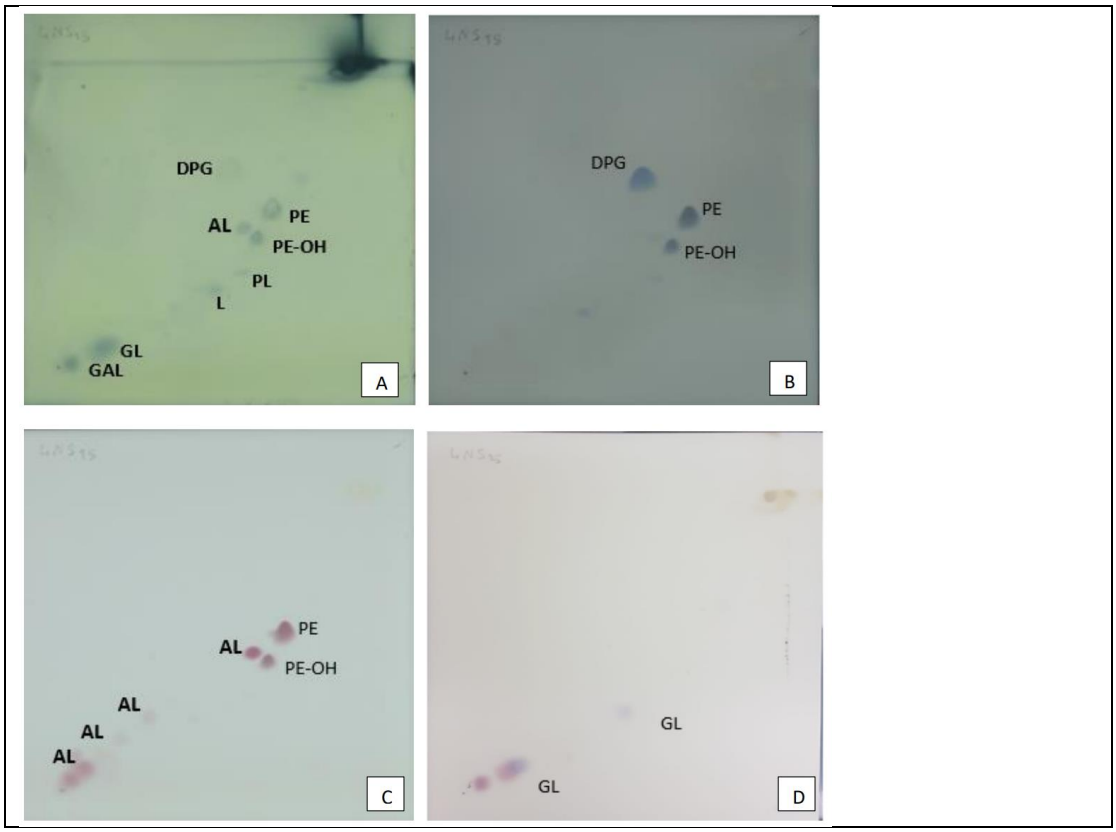


Figure S17 Two-dimensional thin-layer chromatogram of the polar lipids from strain 4NS15T detected with reagents: molybdophosphoric acid reagent (A), molybdenum blue spray reagent (B), ninhydrin reagent (C), a-naphthol reagent (D). DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PE-OH, phosphatidylhydroxyethanolamine; AL, aminolipid; GAL, glycoaminolipid; PL, phospholipid; GL, glycolipid and L, lipid.

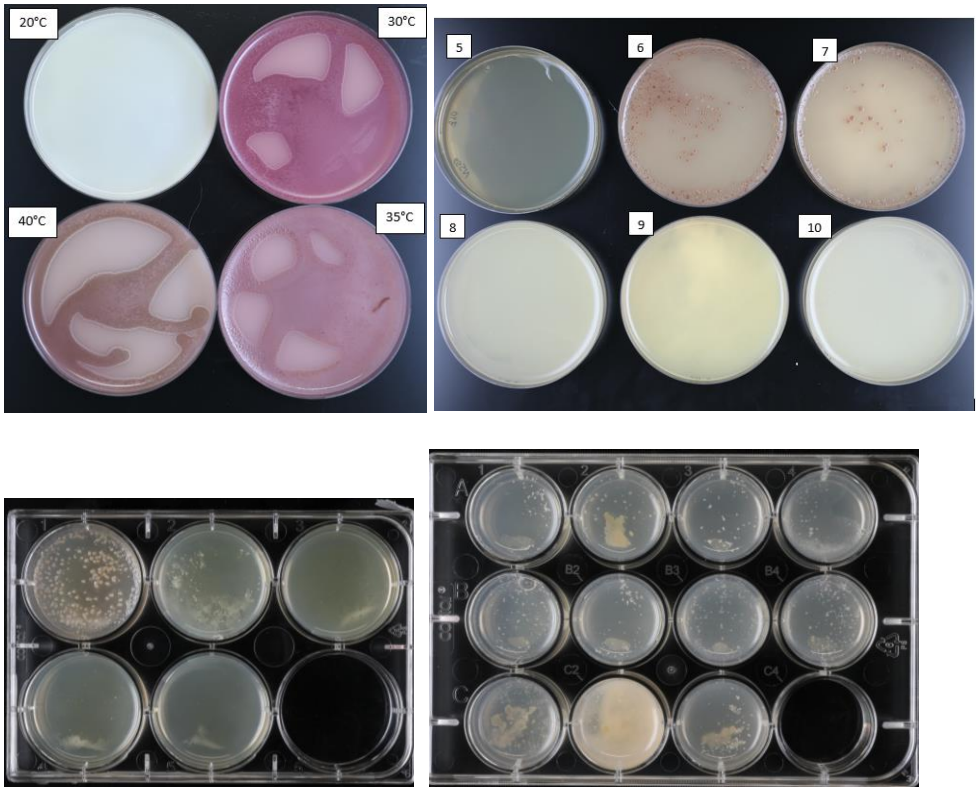


Figure S18 Results of temperature, pH, salt and carbon utilisation of strain NS59

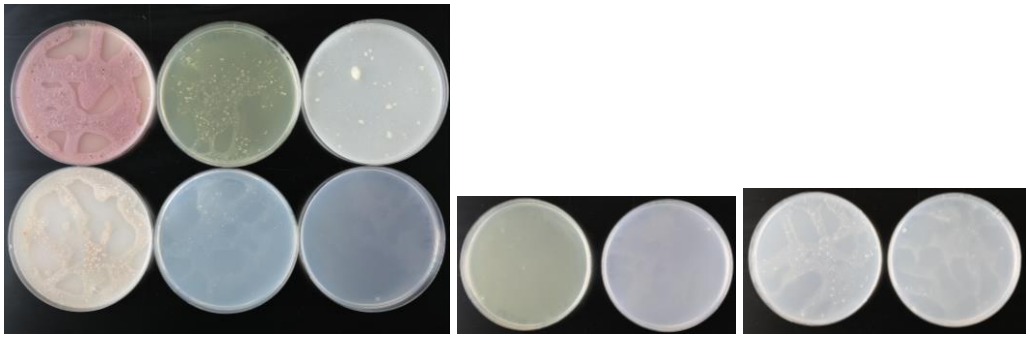
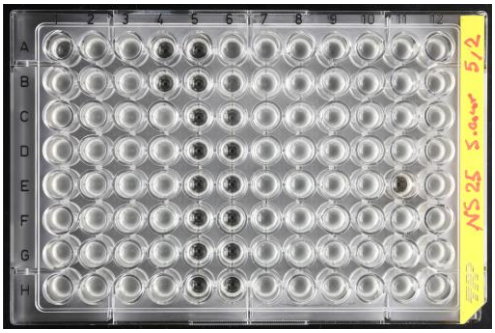
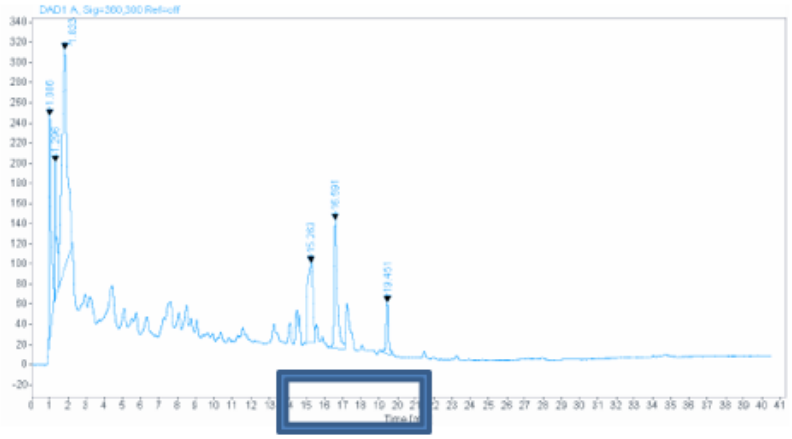


Figure S19 ISP plates of Strain NS59 (left), ISP 6,7 (middle), SSM+/- (right)

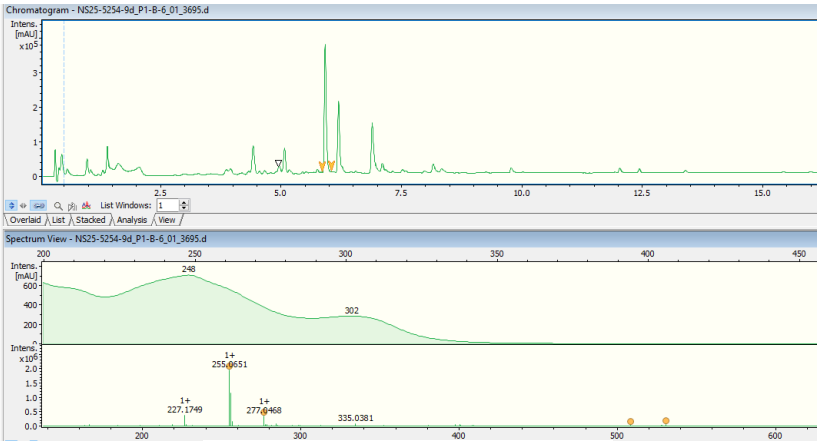
A



B



C



D

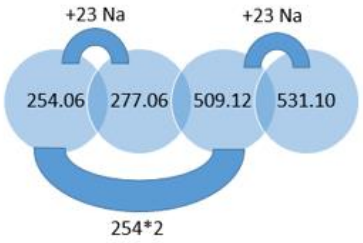
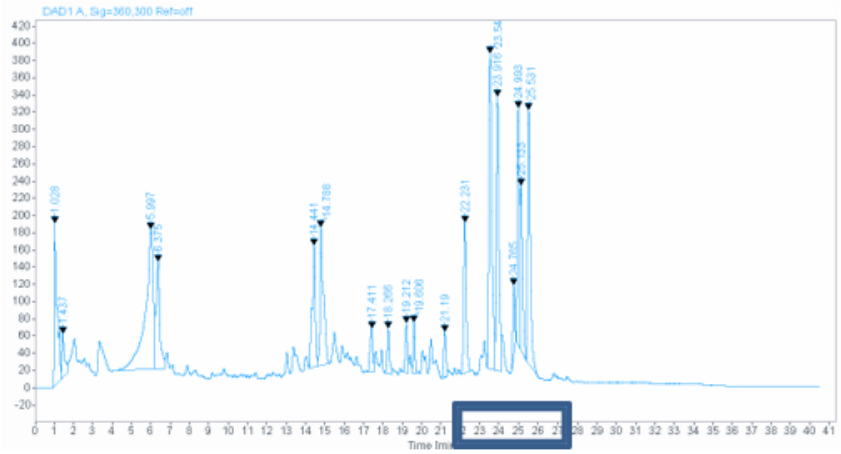


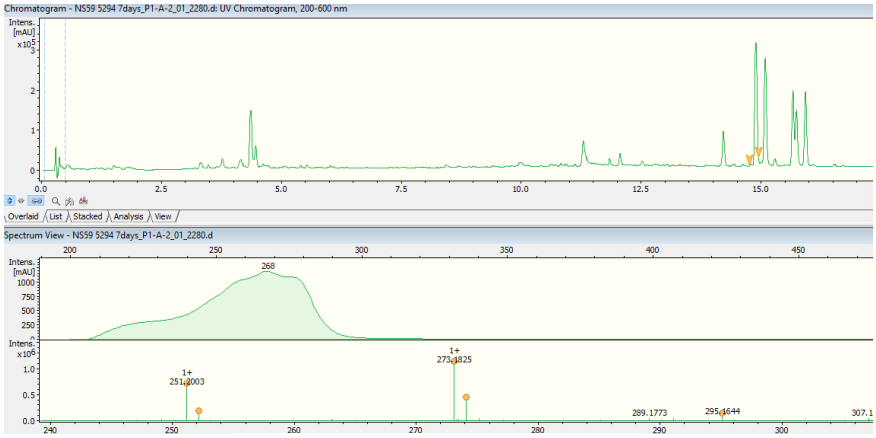
Figure S20 Strain NS25, A) Fractionation result by HPLC; B) HPLC chromatogram and active zone; C) LC-MS spectra; D) Correlated masses of identified family of compounds

A

B



C



D

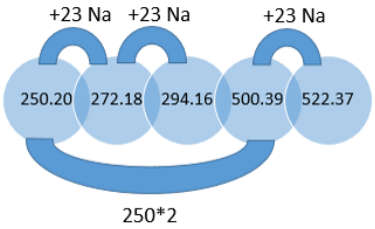


Figure S21 Strain NS59, A) Fractionation result by HPLC; B) HPLC chromatogram and active zone; C) LC-MS spectra; D) Correlated masses of identified family of compounds

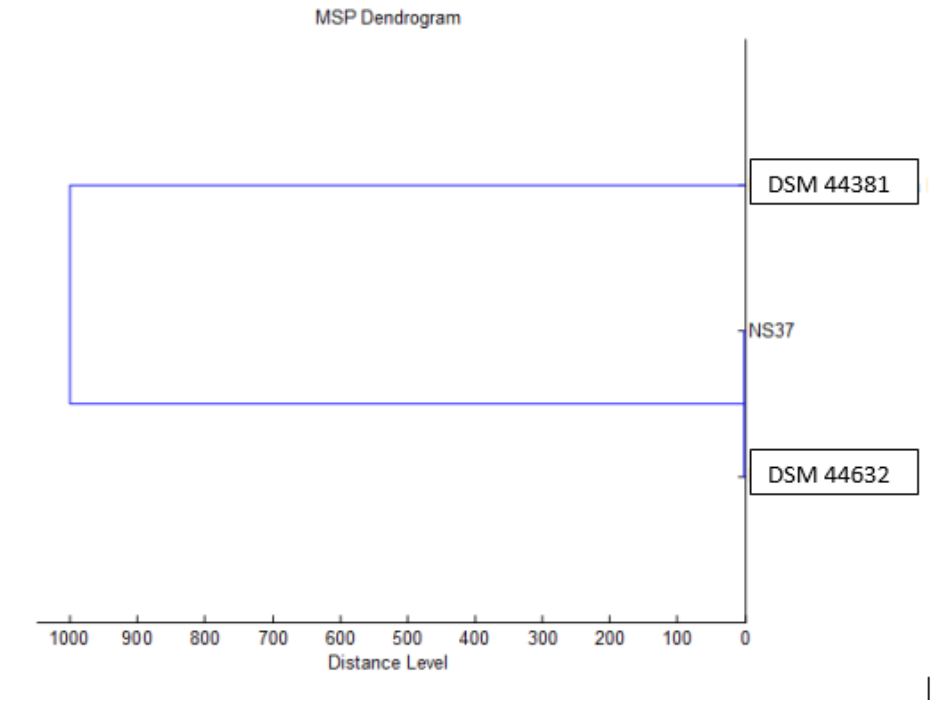


Figure S22 Dendrogram obtained by cluster analysis of MALDI-TOF MS spectra of NS37

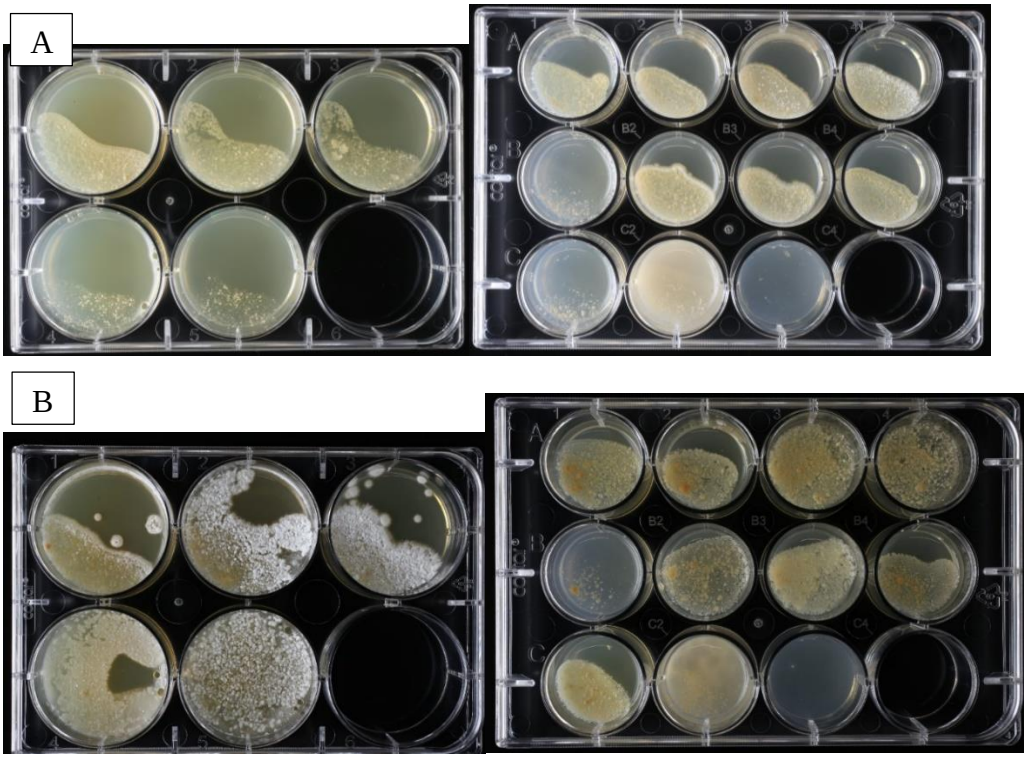


Figure S23 Salt and carbon utilisation of strains A) NS40 and B) NS37

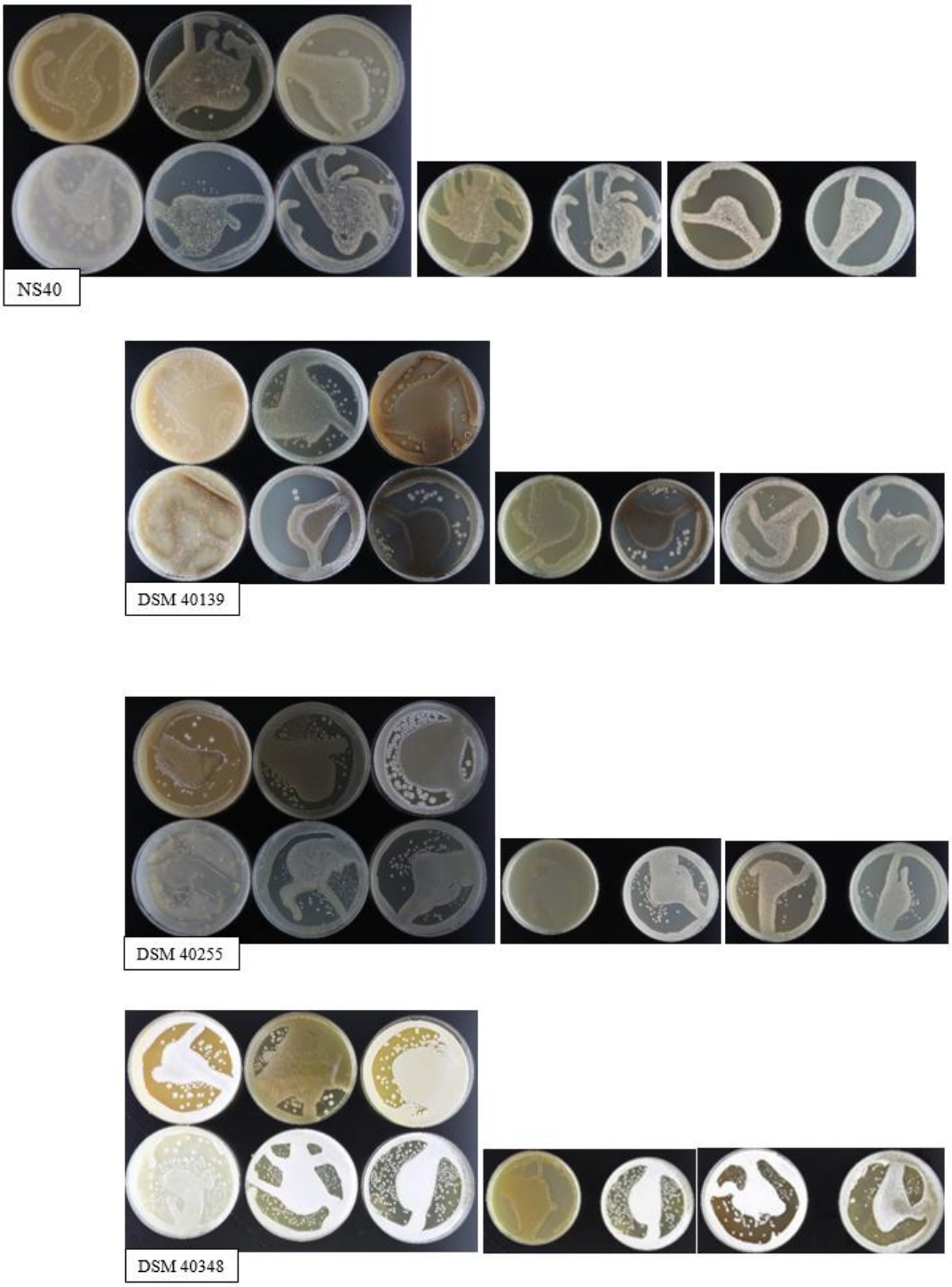


Figure S24 ISP plates of NS40 and type strains (left) A)GYM, B)ISP2, C)ISP3, D)ISP4, E)ISP5, F)ISP7 (right) A)ISP6, B)ISP7, C)SSM+, D)SSM-

Figure S25 ISP plates of NS37 and type strains (left) A)GYM, B)ISP2, C)ISP3, D)ISP4, E)ISP5, F)ISP7

Figure S26 Enzyme activity of strain NS40 and related type strains

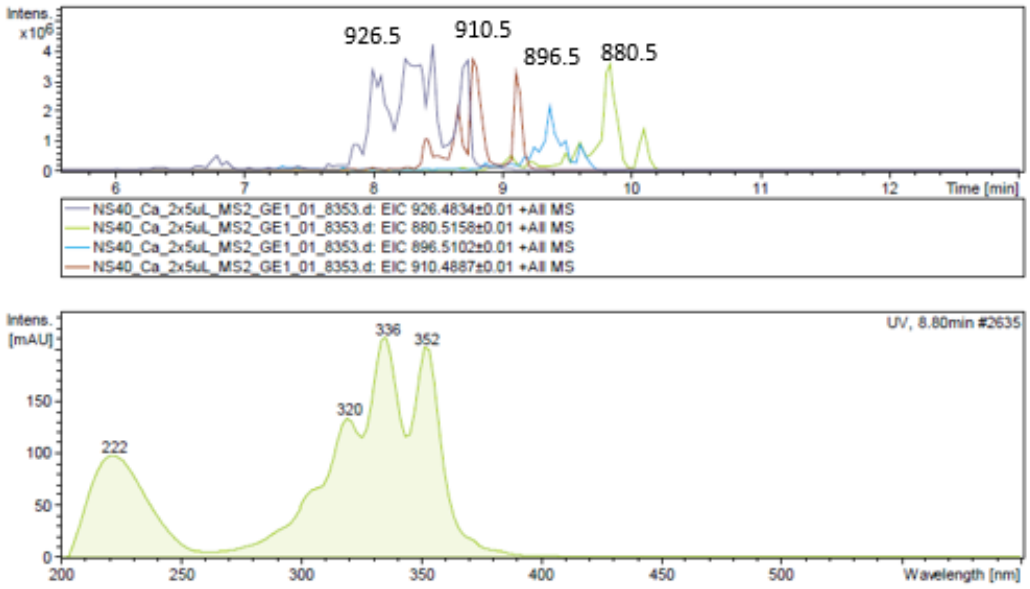


Figure S27 LC–MS spectra of the family of compounds in active area with specific UV absorption at 222, 320, 336 and 352 nm.

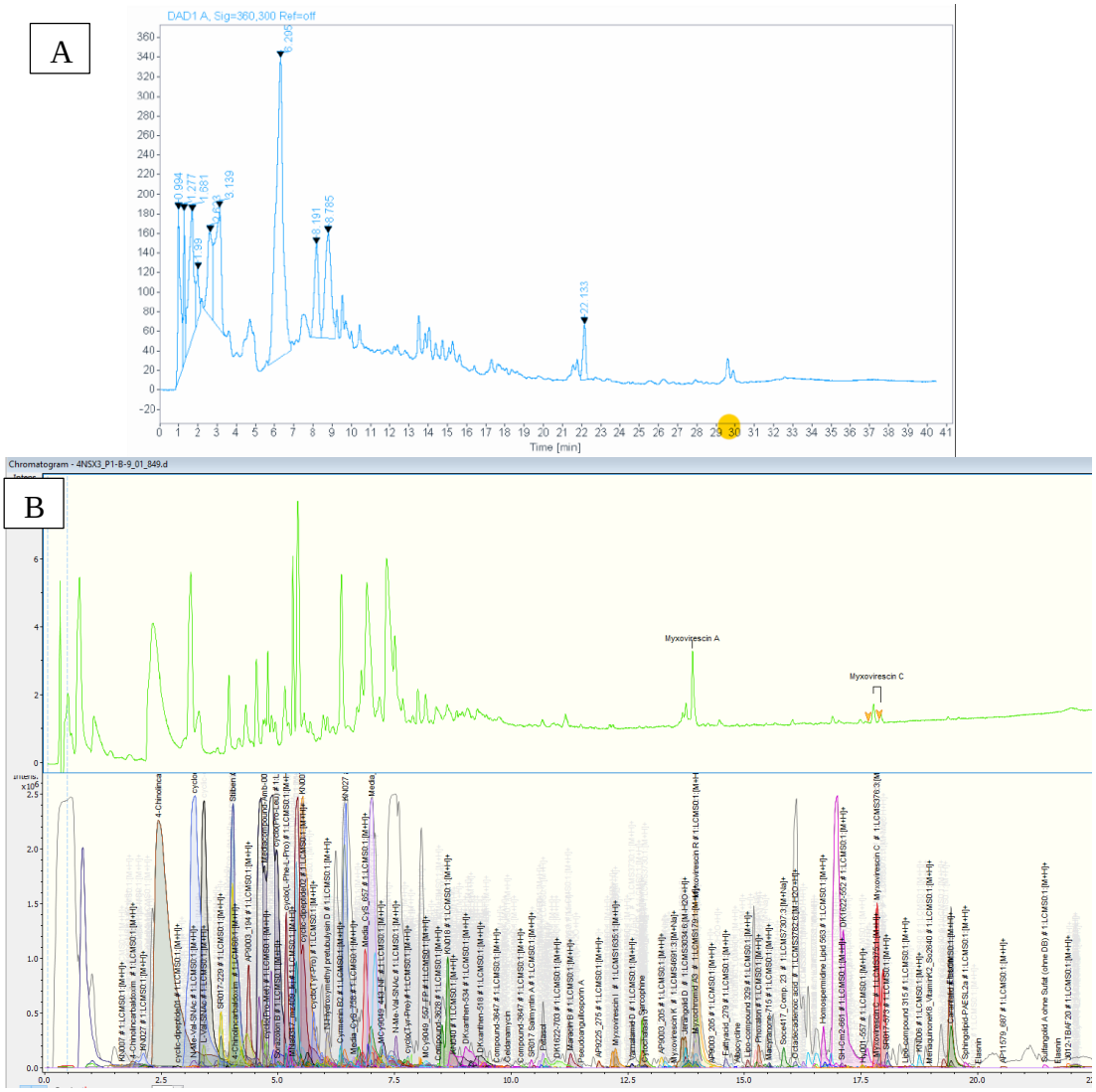
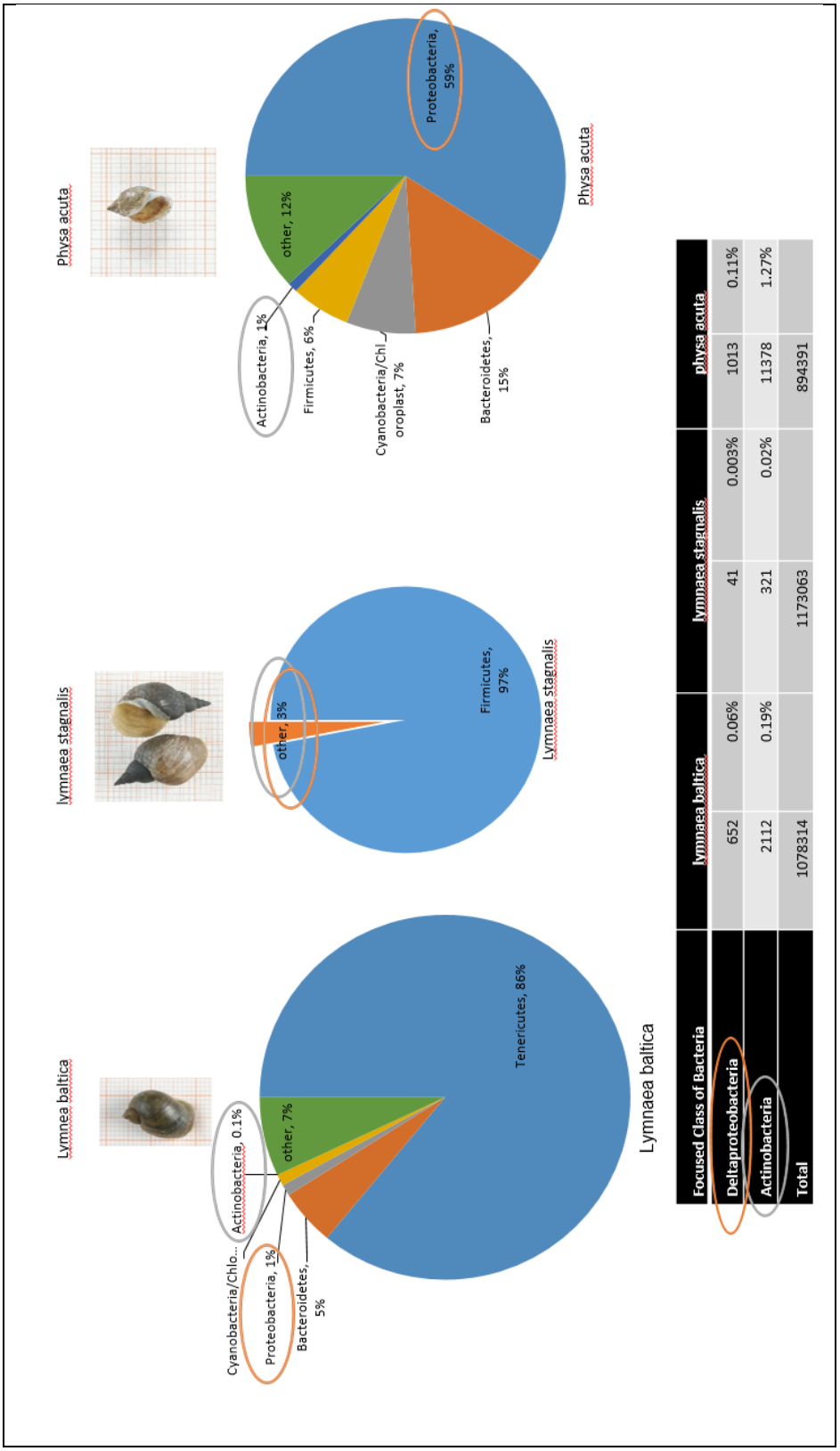


Figure S28 A) Active zone in HPLC chromatogram, B) LC-MS spectra with identified compound



Focused Class of Bacteria	lymnaea baltica		lymnaea stagnalis		physa acuta	
Deltaproteobacteria	652	0.06%	41	0.003%	1013	0.11%
Actinobacteria	2112	0.19%	321	0.02%	11378	1.27%
Total	1078314		1173063		894391	

Figure S29 Bacterial taxonomic profile of three freshwater snails of *Lymnaea baltica, Lymnaea stagnalis* and *Physa acuta* microbiome at the phylum level

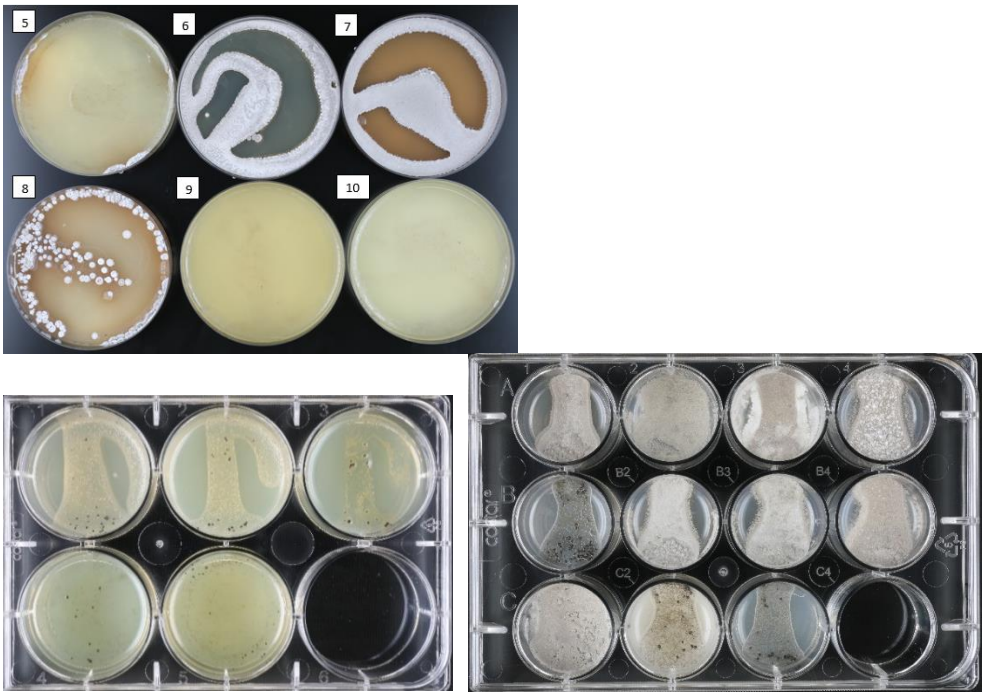


Figure S30 Results of pH, salt and carbon utilisation of 7NS3

Figure S31 ISP plates of strain 7NS3

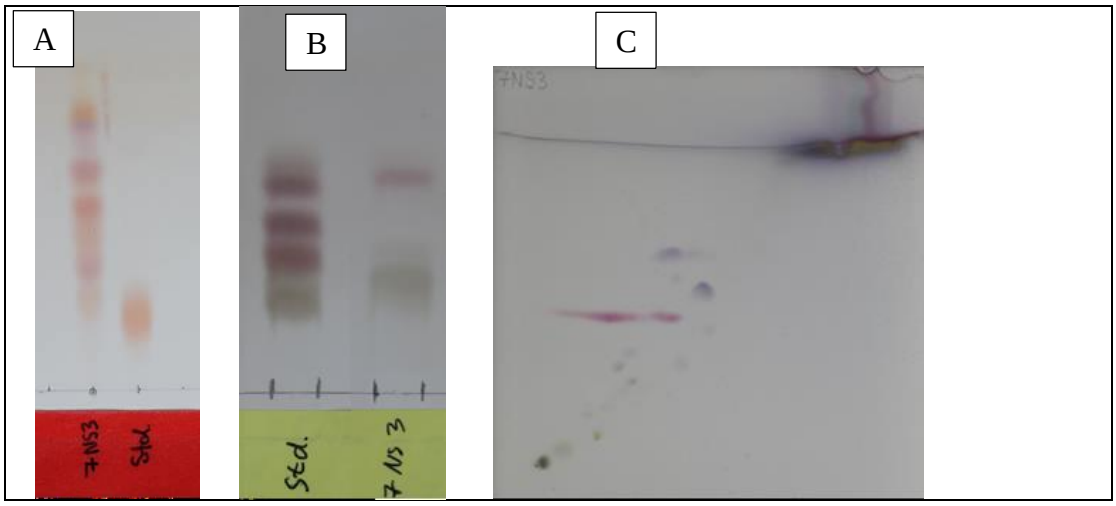


Figure S32 Results of identified amino acids, sugars and polar lipids in strain 7NS3 (A= Amino acid TLC plate, B= Sugar results, C= Polar lipids TLC plate)

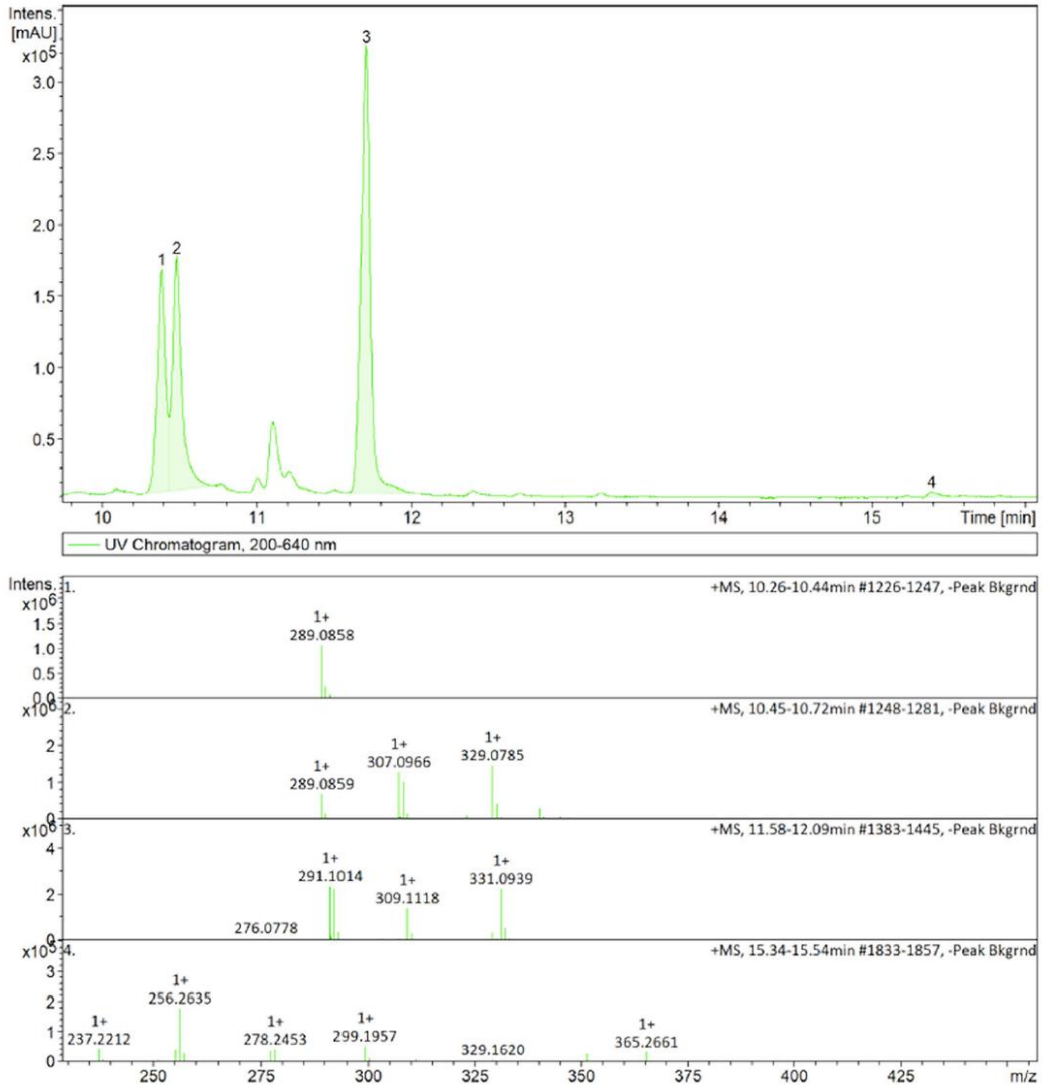


Figure S33 Extracted ion chromatogram 200–640 nm for peaks 1– 4. 1: *m/z* 289.0858 $(M+H)^+$, retention time (t_R) = 10.39 min, 2: *m/z* 307.0966 $(M+H)^+$, *m/z* 329.0785 $(M+Na)^+$, *m/z* 289.0859 $(MH^+–H_2O)$, t_R = 10.49 min, 3: *m/z* 309.1118 $(M+H)^+$, *m/z* 331.0939 $(M+Na)^+$, *m/z* 291.1014 $(MH^+–H_2O)$, t_R = 11.71 min and 4: *m/z* 256.2635 $(M+H)^+$, *m/z* 278.2453 $(M+Na)^+$, *m/z* 237.2212 $(MH^+–H_2O)$, t_R = 15.39 min.

Region	Type	From	To	Most similar known cluster		Similarity
Region 2.1	NRPS-like	72	31,567	stenothricin	NRP:Cyclic depsipeptide	13%
Region 2.2	T3PKS	210,892	250,828	alkylresorcinol	Polyketide	100%
Region 2.3	NRPS, T3PKS	333,972	434,416	scabichelin	NRP	100%
Region 3.1	lassopeptide	44,881	67,030			
Region 3.2	lanthipeptide, bacteriocin	108,242	135,416	informatipeptin	RiPP:Lanthipeptide	100%
Region 5.1	siderophore	56,673	68,634			
Region 5.2	NRPS, T1PKS, terpene	181,761	279,623	naphthyridinomycin	NRP	7%
Region 6.1	T2PKS, siderophore	159,682	232,476	lugdunomycin	Polyketide	44%
Region 6.2	terpene	312,188	334,347	geosmin	Terpene	100%
Region 6.3	bacteriocin	344,596	355,909			
Region 7.1	terpene	57,132	78,214	albaflavenone	Terpene	100%
Region 8.1	terpene	228,203	249,093	BD-12	NRP	17%
Region 10.1	siderophore	61,405	73,174	desferrioxamin B / desferrioxamine E	Other	66%
Region 14.1	ectoine	63,866	74,270	ectoine	Other	100%
Region 15.1	T2PKS	58,891	131,396	spore pigment	Polyketide	83%
Region 17.1	NRPS	41,793	96,219	ibomycin	Polyketide	7%
Region 18.1	melanin	123,893	134,438	melanin	Other	60%
Region 24.1	LAP, thiopeptide	47,415	82,274	thiotetroamide	Polyketide	11%
Region 40.1	terpene	1	13,551	hopene	Terpene	61%

Figure S34. List of predicted secondary metabolite gene clusters for strain 7NS3 identified by analysis of the 7NS3 genome sequence with the bioinformatic tool antiSMASH 5.0